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The present proceedings of the U.S. Environmental Protection Agency's Second Symposium on the Application of Short-term Bioassays in the Fractionation and Analysis of Complex Environmental Mixtures, held in Williamsburg, VA, March 4-7, 1980, includes 37 papers as well as the Keynote Address. The papers are divided according to the environmental media wherein short-term bioassays are applied--ambient air, water, and soil--and the sources of environmental pollution--mobile source emissions, stationary source emissions, and industrial emissions and effluents. A separate section is devoted to the problems of health hazard and risk assessment.

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FOREWORD

The many benefits of our modern industrial society are accompanied by certain hazards. Careful assessment of the relative risk of existing and new man-made environmental hazards is necessary for the establishment of sound regulatory policy. These regulations serve to enhance the quality of our environment in order to promote the public health and welfare and the productive capacity of our nation's population.

The Health Effects Research Laboratory, Research Triangle Park, North Carolina, conducts a coordinated environmental health research program in toxicology, epidemiology, and clinical studies using human volunteer subjects. These studies address problems in air pollution, non-ionizing radiation, environmental carcinogenesis, and the toxicology of pesticides as well as other chemical pollutants. The Laboratory develops and revises air quality criteria documents on pollutants for which national ambient air quality standards exist or are proposed, provides the data for registration of new pesticides or proposed suspension of those already in use, conducts research on hazardous and toxic materials, and is preparing the health basis for non-ionizing radiation standards. Direct support to the regulatory function of the Agency is provided in the form of expert testimony and preparation of affidavits as well as expert advice to the Administrator to assure the adequacy of health care and surveillance of persons having suffered imminent and substantial endangerment of their health.

The Second Symposium on the Application of Short-term Bioassays in the Fractionation and Analysis of Complex Environmental Mixtures arose out of the recent developments in the methodology for the chemical analysis and bioassay of complex environmental mixtures from a variety of media. The present proceedings reflects the state-of-the-art in this promising area of research for the identification and evaluation of potential human health hazards.

F. Gordon Hueter, Ph.D.
Director
Health Effects Research Laboratory

ABSTRACT

The present proceedings of the U.S. Environmental Protection Agency's Second Symposium on the Application of Short-term Bioassays in the Fractionation and Analysis of Complex Environmental Mixtures, held in Williamsburg, VA, March 4-7, 1980, includes 37 papers as well as the Keynote Address. The papers are divided according to the environmental media wherein short-term bioassays are applied--ambient air, water, and soil--and the sources of environmental pollution--mobile source emissions, stationary source emissions, and industrial emissions and effluents. A separate section is devoted to the problems of health hazard and risk assessment.

DISCLAIMER

This report has been reviewed by the Health Effects Research Laboratory, U.S. Environmental Protection Agency, and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

PREFACE

More than one hundred short-term bioassays are now available for detecting the toxicity, mutagenicity, and potential carcinogenicity of chemicals. These bioassays were developed and validated with individual compounds, and their principal application was perceived to be in evaluating the health hazard of such materials. However, man is rarely exposed to single chemicals; his exposure to hazardous chemicals is more commonly a multifactorial phenomenon. Although chemical analysis can be used to detect known hazardous compounds, it would be a staggering and expensive task to analyze large numbers of samples for all known or suspected hazardous constituents. Furthermore, the biological activity of a complex mixture cannot be reliably predicted from knowledge of its components. On the other hand, bioassays alone cannot tell us which components of complex mixtures are responsible for the biological activity detected. Thus, cost effectiveness and technical feasibility dictate stepwise and perhaps iterative application of both chemical and biological methods in evaluating the health effects of complex environmental mixtures.

Through the coupling of reliable biological detection systems with methods of chemical fractionation and analysis, it is frequently possible to isolate the individual chemical species that show biological activity. Initially, complex mixtures may be separated and bioassayed in carefully defined chemical fractions. The results of such short-term screening bioassays then may be used to guide the course of further fractionation and to determine the need for more stringent and comprehensive biological testing.

Another approach to the screening of complex environmental mixtures for health effects involves the use of *in situ* bioassays. The biological effects of environmental chemicals are influenced by a combination of environmental factors that cannot be completely reproduced in the laboratory. By maintaining test organisms at sites to be monitored, one can rapidly identify potentially hazardous environmental mixtures that warrant further investigation.

These are the proceedings of the Second Symposium on the Application of Short-term Bioassays in the Fractionation and Analysis of Complex Environmental Mixtures, held in Williamsburg, VA, March 4 through 7, 1980, and sponsored by the U.S. Environmental Protection Agency. The first symposium of this series, also held in Williamsburg, in February, 1978, combined accounts of the latest methods for collection and chemical analysis of complex samples with discussions of current research involving the use of short-term bioassays in conjunction with fractionation and analysis of such mixtures. The emphasis of the present proceedings is on the application of these methods in testing a variety of media, including ambient air, drinking water and aqueous effluents, terrestrial systems, mobile-source emissions, and stationary-source emissions and effluents. The critical problem of human health hazard and risk assessment is also addressed.

We hope that this volume will help to consolidate our knowledge of the techniques and applications of chemical analysis and bioassay of complex environmental mixtures and that it will provide direction for further research in this area.

Michael D. Waters
Shahbeg S. Sandhu

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KEYNOTE ADDRESS

KEYNOTE ADDRESS

Vilma Hunt
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I am delighted to be here today addressing this symposium on the application of short-term bioassays to the study of complex environmental mixtures. I can think of no other area that is more representative of the significant advances that have been made in the last decade in the environmental health sciences field.

The U.S. Environmental Protection Agency (EPA) has been faced since its inception with the responsibility to control harmful environmental compounds. One of the highest priorities of EPA's research and development program is the protection of health through the identification and control of toxic substances. Our responsibility in the Office of Health Research is to identify, qualitatively and quantitatively, the harmful health effects associated with environmental agents. In our effort to fulfill this responsibility, we have repeatedly been slowed by the limitations of the available testing procedures, as well as by a relative lack of understanding of health effects themselves.

One of the major problems we have faced and still face is the complexity of the agents of concern. Complex mixtures--whether industrial effluents or emissions, or ambient environmental media--are generally entities composed of hundreds of compounds. Further, these mixtures are often poorly defined and of continuously changing chemical composition. As such, complex mixtures present special challenges in quantitation and assessment that require considerable effort to overcome.

We are also faced with limitations in our understanding of the effects we must test for. The effects of concern--cancer, mutagenesis, teratogenesis, and other toxic impacts--are often

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chronic and slow to develop. The science behind these effects is not well understood. And, significantly, we are often looking for effects that are expected to be of low probability for an individual, but of large impact for the exposed population.

The magnitude of the environmental regulatory task that EPA and other regulatory agencies face is underscored by the astronomical numbers that come up whenever environmental assessment is discussed. There are currently more than 4,000,000 known chemical compounds; thousands more are being discovered each year; 70,000 are in common use, produced and distributed by some 115,000 industries and firms. Billions of gallons of industrial effluents are discharged into our lakes, rivers, and oceans each year. The 1977 emission of criteria air pollutants to the atmosphere was 190 million tons, a quantity that does not include the other unregulated and potentially hazardous particles, gases, and aerosols emitted each year. In addition, several billion tons of unwanted solid waste--some harmful, some innocuous--are disposed of each year.

If, to fulfill our health effects assessment responsibility, we had "only" to establish the toxic potential of some 70,000 compounds, we would be faced with a staggering assignment. When consideration is given to the fact that these compounds appear in the environment in diverse combinations, in a variety of media, and often in miniscule quantities that are difficult to collect and analyze, the staggering assignment suddenly appears overwhelming.

Our efforts to assess toxic potential include a number of undertakings in laboratory, clinical, and epidemiological research. Historically, EPA and other government regulatory agencies have favored established whole-animal methods as the standard of reference to establish carcinogenicity and other toxic effects. Our resources, however, to conduct whole-animal studies are limited. Indeed, the world laboratory capacity for conducting these experiments has been estimated at 500 compounds per year, a small number when compared with the number of compounds needing assessment. Additionally, the long-term nature of whole animal studies poses other problems for regulators faced with the need to make timely regulatory decisions.

Data derived from epidemiological studies are, of course, those most adequate from a regulatory standpoint. These data are scarce, however, and we are again faced with fiscal limitations in collecting sufficient information on large numbers of compounds, not to mention the inherent difficulties in collecting meaningful information for large populations exposed to a myriad of compounds.

Recognizing our limitations in whole-animal and epidemiological studies, we have devoted particular attention and considerable resources to the research and development of short-term tests over the last several years. Our interest in short-term bioassays was sparked by recognition of their potential value to a health effects assessment program. Our investment has been rewarded by highly encouraging results.

As a rapid, effective, and inexpensive means to identify the impact of complex mixtures, short-term testing can play a critical role in the monitoring of the environmental media for presumptive health hazards. Today, any program aimed at identifying and reducing production and release of large numbers of hazardous agents must take advantage of short-term bioassays to set priorities for further evaluation by conventional toxicological, clinical, and epidemiological investigations. By efficiently using short-term bioassays and through the development of approaches that combine the use of various bioassay systems, we have begun to screen large numbers of potentially harmful compounds in a systematic and effective manner.

As this symposium reflects, the application of short-term bioassays to the assessment of complex mixtures has been developed hand-in-hand with the application of state-of-the art analytical chemistry techniques. The iterative application of chemical and biological analytical tools has greatly expanded the number of environmental pollutants for which biological hazards have been identified. Results of many of these studies will be reported on during the next several days. However, the number of assessed pollutants is still only a small fraction of those requiring assessment. The lack of information on chemical composition and biological activity continues to constitute a major barrier to the assessment of human health hazards from complex environmental mixtures.

At the first symposium on The Application of Short-term Bioassays in the Fractionation and Analysis of Complex Environmental Mixtures, Dr. Michael Waters of our Research Triangle Park Laboratory dedicated the meeting "to the concept that the joint application of state-of-the art biological and chemical analytical techniques is the appropriate approach in environmental research." I am happy to note that the multidisciplinary approach being pursued in this area is representative of a widespread evolution in the approach to environmental problems on many fronts. We are moving away from the segmented, narrow approaches of the past to joint undertakings that direct the activities of different disciplines toward specific, unified goals.

The past two years have seen great strides in the short-term bioassay field and in the analytical area. The symposium last year was devoted to the "nuts and bolts" of this relatively new field. The majority of the presentations dealt with the bioassay techniques, the sampling methods, and the analytical procedures. This year, the symposium is organized to report on the application of these techniques--a reflection of the development of this field from solely an object of research to a means for research.

In particular, the topics to be considered over the next several days include the utilization and application of short-term testing to different media: ambient air, drinking water and effluents, terrestrial media, mobile-source emissions, and stationary-source emissions and effluents. The Friday morning session is devoted to an examination of the role of short-term testing in hazards assessment, a topic of concern and interest to all of us in the post-Love Canal era when we are confronted with resolving the buried mistakes of the past.

In addition to an increase in the application and utilization of short-term procedures, the past two years have also been marked by the validation of many of the short-term bioassay procedures. The validation of short-term testing was essential if short-term testing was to find a meaningful role in environmental health assessment. Considerable effort has been devoted to developing the needed data, and although much work remains to be done, the initial results are optimistic. EPA recently established the Gene-Tox program to further the evaluation and validation of short-term bioassays. This program, which is evaluating 27 different short-term systems, will play an important role in identifying aspects of short-term tests that require further development and validation. Information from the Gene-Tox evaluations will be used to direct future research programs.

In conclusion, the coupling of short-term bioassays with state-of-the-art chemical analysis techniques is an exciting and rapidly evolving field--one that offers the potential to resolve questions of nontoxicity quickly and to provide a scientific basis for properly allocating our resources among many studies of potentially hazardous agents. If the past is an augury of the future, the challenges that face this still young and rapidly growing field will be met. I am enthusiastic about the recent developments in this field and the developments that are forthcoming. The importance of this research to the future of environmental health assessment cannot be overstated.

Thank you.

SESSION 1

AMBIENT AIR

PB82-233180

BIOASSAY OF PARTICULATE ORGANIC MATTER FROM AMBIENT AIR

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INTRODUCTION

The influence of industrialization and consequent increased concentration of urban particulate matter on the incidence of cancer has long been a concern (Kotin and Falk, 1963; Carnow and Meier, 1973). The first bioassays used to evaluate complex ambient air samples were whole-animal carcinogenesis bioassays (Leiter et al., 1942; Hueper et al., 1962). In these studies, organic extracts of urban particulate matter were found to be carcinogenic in rodents. Such organic extracts have also been shown to transform rodent embryo cells in culture (Freeman et al., 1971; Gordon et al., 1973). Carcinogenic polycyclic aromatic hydrocarbons (PAH), such as benzo(a)pyrene, were detected in these extracts; however, these compounds did not account for all of the carcinogenic activity reported.

The development of the Ames Salmonella typhimurium mutagenesis bioassay (Ames et al., 1975) provided a simpler, more sensitive, and faster bioassay for potential carcinogenic activity that could be applied to air samples collected by conventional techniques. The initial applications of this bioassay to ambient air particulate organic matter (Tokiwa et al., 1976; Pitts et al., 1977; Talcott and Wei, 1977) stimulated research in the following areas:

1) improvement in sample collection, extraction, and bioassay methodology;

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2) characterization and identification of potential classes of carcinogens and specific carcinogens present in ambient air particles; and

3) evaluation of emission sources and atmospheric conditions responsible for the observed mutagenicity in urban air particulate.

This overview addresses these areas of research and summarizes our current understanding of the mutagenicity of particulate organic matter found in ambient air.

ADVANCES IN SAMPLE COLLECTION AND EXTRACTION

The first reported studies on the mutagenicity of particulate organic matter from ambient air used high-volume samplers to collect air particles on glass fiber filters (Tokiwa et al., 1976; Pitts et al., 1977; Talcott and Wei, 1977). This sampler collects both respirable particles (< 5 μm) and non-respirable large particles. In these studies, the organics were extracted from the particles with either methanol (Tokiwa, 1976), acetone (Talcott and Wei, 1977), or a mixture of methanol, benzene, and dichloromethane (1:1:1)(Pitts et al., 1977). Since high-volume samplers are widely used in air-monitoring programs to determine total suspended particulate (TSP) levels, these studies provided comparative mutagenicity data for different sites.

Although high-volume samplers provide the simplest, and for many investigators the only, method available for collection of air particles, this method presents several serious disadvantages. The most consequential disadvantage is that respirable particles are collected simultaneously with larger particles. In cases where the smaller respirable particles are considerably more mutagenic than the larger particles, the larger particles dilute the overall mutagenicity of the sample, thereby biasing the analysis of the particle composition to which the human lung is exposed.

Other potential disadvantages are due to the large volume of air being drawn continuously over collected particles. Samples collected by this method may lose more volatile organics by evaporation. The organics present on the particles are also potentially subject to reactions with nitrogen dioxide (NO_2), ozone (O_3), or peroxyacetyl nitrate (PAN), which are all present in urban air. Pitts et al. (1978a, b) have shown that PAH (e.g., benzo(a)pyrene and perylene) directly coated onto glass fiber filters reacted with NO_2, O_3, and PAN, as well as ambient photochemical smog, to form several direct-acting mutagens (mutagens that do not require an exogenous microsomal activation system). Although these reactions have not been shown to occur to

PAH adsorbed on the surface of air particles, potential surface reactions during filtration still must be taken into consideration when interpreting studies using filtration for sample collection.

A recent modification of the standard high-volume sampler, the size-selective inlet (SSI) high-volume sampler, collects only particles < 15 μm (the definition of "inhalable particles"). This sampler excludes the larger particles, which are not normally inhaled.

A sampling device that does collect particles in separate size fractions is the cascade impactor (Andersen, 1966). These samplers use a series of plates with either holes or slots offset at each stage to collect separate size fractions ranging from < 2 μm to > 7 μm. The cascade impactor is generally attached to a high-volume sampler that collects the smallest particles by filtration. Teranashi et al. (1977), using an Andersen high-volume cascade impactor, found that the organics from particles < 1.1 μm, collected on the backup filter, were significantly more mutagenic than the organics from the larger particles. Pitts et al. (1978b) reported similar findings using a Sierra high-volume cascade impactor in downtown Los Angeles. This method, while providing a size-fractionated sample for bioassay, still employs filtration to collect the smallest particles, which contain most of the mutagenic components.

In order to collect larger quantities of size-fractionated air particulate matter for biological studies, the U.S. Environmental Protection Agency (EPA) had a Massive Air Volume Sampler (MAVS) designed and fabricated by Henry and Mitchell (1978) that does not require filtration. The MAVS employs two impactors that collect 3.5- to 20-μm and 1.7- to 3.5-μm particles, followed by an electrostatic precipitator (ESP) that collects the particles < 1.7 um. In our initial studies with the MAVS at a Los Angeles freeway site, we found the particles with mean diameters < 1.7 μm to be significantly more mutagenic than the larger particles (Figure 1). However, when the ESP was charged to maximize collection efficiency, as much as 0.05 ppm of O_3 was measured at the blower outlet of the sampler (Mitchell et al., 1978). Jungers et al. (1980) evaluated the effect of O_3 under the MAVS operating conditions on both the mutagenicity and chemical composition of the particulate organic matter collected in the ESP. These studies found that under these operating conditions, the O_3 did not significantly affect the mutagenicity or the PAH content of the organics.

A wide range of extraction methods have been used to remove the organics from air particles for bioassay. Solvents employed range from the nonpolar solvents cyclohexane (Mϕller and Alfheim, 1980) and benzene (Teranashi et al., 1977) to acetone (Talcott and Wei, 1977) and the more polar solvent methanol (Tokiwa et al.,

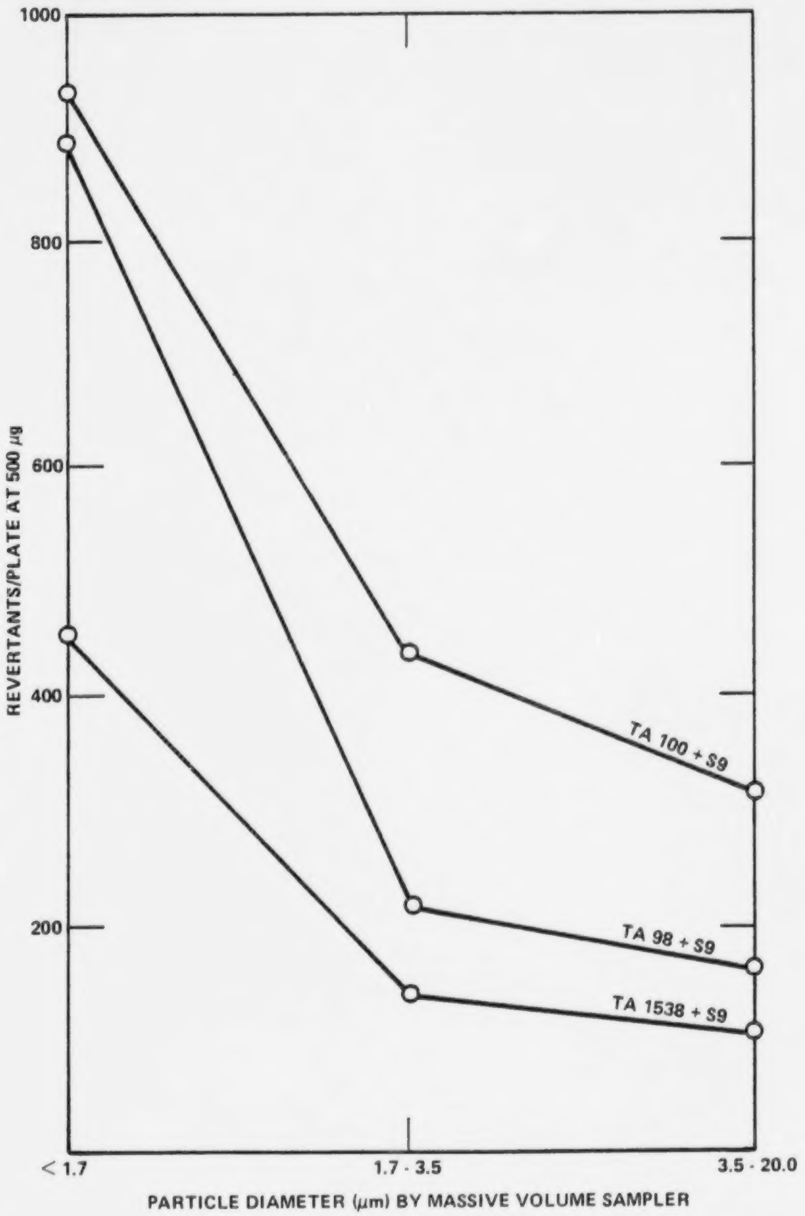

Figure 1. Mutagenicity of air particulate at the Los Angeles freeway site (upwind) as a function of particle size.

1976). Since different solvents will preferentially remove different constituents from the particles, the method of extraction can significantly alter the resulting composition and mutagenicity of the organics. Jungers et al. (1980), after evaluating seven solvent systems, found that dichloromethane extraction resulted in the most mutagenic extractible organics with a minimum of inorganic anions present.

CHARACTERIZATION AND IDENTIFICATION OF POTENTIAL CARCINOGENS

The application of the Ames *S. typhimurium* plate-incorporation assay (Ames et al., 1975) with multiple tester strains, used in the presence and absence of a metabolic activation system, provides an initial characterization of potential carcinogens present. The organics from air particles generally show mutagenic activity only in the tester strains susceptible to frameshift mutations (e.g., TA1538, TA1537, TA98, and TA100, but not TA1535). Mutagenic activity is usually observed in the absence of a metabolic activation system, indicating the presence of direct-acting mutagens. When metabolic activation is added, certain air samples show significant increases in mutagenicity (Talcott and Wei, 1977; Pitts et al., 1977), while other samples show no increase in activity, and even occasionally a decrease (Talcott and Wei, 1977). Upon fractionation, however, air samples generally have been shown to contain both direct-acting mutagens and mutagens that require the addition of metabolic activation. Table 1 shows the response of five *S. typhimurium* tester strains to a typical air sample, with and without metabolic activation, using the < 1.7-μm particles collected with a MAVS in Birmingham, AL.

Table 1. Specific Mutagenic Activity of Air Particulate Extract (< 1.7 μm)

	Revertants/100 μg Organics	
Tester Strain	Without S-9 Activation	With S-9 Activation
TA1538	81	121
TA1537	59	34
TA98	121	188
TA100	176	245
TA1535	-	-

Bioassay-directed chemical fractionation is an increasingly powerful tool for the identification of potential carcinogens in complex mixtures. This technique has been employed to characterize and eventually identify potential carcinogens in cigarette smoke (Swain et al., 1969), synthetic fuels (Epler, 1980), and diesel emissions (Huisingh et al., 1978). Until the recent development and improvement of air particle collection techniques, such studies on particulate organic matter in air were severely limited by the amount of sample collected. In spite of these difficulties, Teranashi et al. (1977) fractionated particulate organic matter from Kobe, Japan, into acidic, basic, aliphatic, aromatic, and oxygenated fractions. The acidic, aromatic, and oxygenated fractions accounted for most of the mutagenic activity of the total sample. Studies by Kolber et al. (1980), who used a somewhat different fractionation method, also showed significant mutagenic activity in those three fractions.

EVALUATION OF EMISSION SOURCES AND ATMOSPHERIC CONDITIONS

Initial comparative studies (Tokiwa et al., 1976; Pitts et al., 1977) showed that air particulate was more mutagenic at industrial and urbanized sites than at rural sites. Recently, Flessel et al. (1980) compared mutagenicity among sites in Contra Costa County, CA, with differing amounts of industrialization and cancer rates. These studies all indicate a higher mutagenic activity in the more urbanized or industrialized sites.

At any one ambient sampling site, the mutagenicity appears to vary significantly over time. A major parameter affecting airborne mutagenicity, identified by Commoner et al. (1978), in a year-long study at a Chicago school site, was the wind direction. In this study, a plot of wind direction versus relative mutagenic activity showed that wind directions of either northwest or east resulted in air particle samples with the greatest mutagenicity. Møller and Alfheim (1980) reported on the mutagenicity of airborne particles from two locations in Oslo over a three-month period. They observed higher mutagenicity in February (i.e., during the heating season) than in March and April. They also reported significant meteorological effects. The mutagenicity, when calculated as revertants per cubic meter of air, was highest on cold clear days with little wind. When revertants per milligram of particulate matter was calculated and compared with meteorological conditions, mutagenicity was found to be high on days with rain or snow, when the total concentration of particles in the air was low. Although studies have been conducted to examine the effect of ultraviolet light (Gibson et al., 1978) and other atmospheric gases and oxidants including O_3, NO_2, and PAN (Pitts et al., 1978a, b) on PAH, the role of these in the mutagenicity of particulate organic matter in ambient air is still uncertain.

In certain cases, studies can be designed to identify specific emission sources that contribute to the mutagenicity of the ambient particulate organic matter. At the Los Angeles freeway site (discussed above), samplers were situated both upwind and downwind from the freeway. Figure 2 shows the comparative mutagenic activity of organics from particles < 1.7 μm collected over the same period. The particles collected downwind from the automobiles and trucks on the freeway were significantly more mutagenic than those collected upwind. Claxton and Huisingh (1980) have shown that the organics from a gasoline catalyst automobile are significantly mutagenic. It is clear from these studies and others (Huisingh et al., 1978; Löfroth, 1980; Alfheim and Møller, 1980) that both gasoline and diesel engine exhaust from automobiles, buses, and trucks contribute to the mutagenicity of ambient air particles.

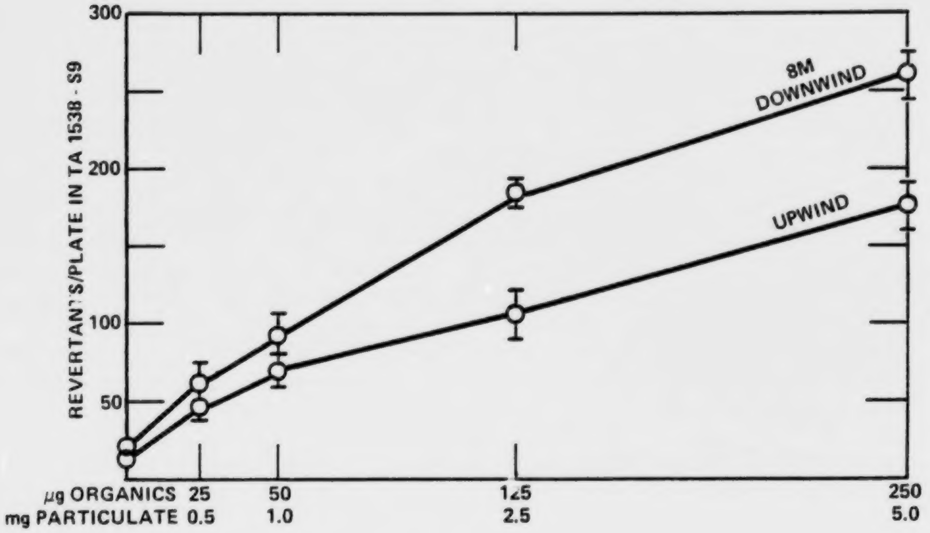

Figure 2. Mutagenicity of air particulate (< 1.7 μm) collected by MAVS upwind and downwind of the Los Angeles freeway.

It is clear that a variety of combustion sources could contribute organic mutagens to the ambient air. Claxton and Huisingh (1980) compared the mutagenic activity of organics from particles emitted from residential heaters as well as diesel and gasoline vehicles. A study by Møller and Alfheim (1980) of two locations in Oslo, Norway, suggested that the mutagenicity they observed was due in part to both automotive traffic and residential heaters, with residential heating probably contributing more in the winter months. Industrial sources of mutagens that may be significant are coke oven emissions (Claxton, 1980) and coal combustion emissions (Chrisp et al., 1978).

SUMMARY

The mutagens present in ambient air particulate possess the following characteristics:

1) They show both direct-acting and indirect-acting mutagenic activity. The proportions of these two classes of activity vary with the sample location.

2) They show mutagenic activity primarily in the tester strains that respond to frameshift mutagens.

3) They appear to be present in higher concentrations in the smallest particles (< 2 μm) than in larger particles.

4) They appear to result from specific emission sources (such as combustion sources).

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COLLECTION, CHEMICAL FRACTIONATION, AND MUTAGENICITY BIOASSAY OF AMBIENT AIR PARTICULATE

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INTRODUCTION

Our industrial society has created thousands of synthetic xenobiotics to support our modern lifestyle. Many of these substances, and their by-products, enter our atmosphere in the form of vapor-phase and particulate pollutants that can be ingested by respiration and skin contact. The insults to human health and the ecosystem from these airborne organic pollutants are due mainly to polycyclic organic matter, specific industrial emissions such as halogenated hydrocarbons, and end-use chemicals such as pesticides (Fishbein, 1976). Polycyclic organics associated with air particulates are believed to result primarily from incomplete combustion of organic matter (Møller and Alfheim, 1980). These carcinogenic components, (benzo(a)pyrene (B[a]P), benz(a) anthracene) and other polycyclics have been well characterized chemically (Shubik and Hartwell, 1969). As population and industrial activity increase, the growing health hazards associated with ambient air pollution must be further assessed and evaluated.

Air pollutants are chemically complex environmental mixtures, whose compositions vary geographically with local industrial activity and weather conditions. Seasonal variations in chemical composition and mutagenicity of organic extracts of air particulate have been documented in Oslo, Stockholm, and New York City (Møller and Alfheim, 1980; Löfroth, 1980; Daisey et al., 1979). All three communities exhibited higher mutagenicity during the winter months, and in each case, a major contributing factor was polynuclear aromatic hydrocarbons (PAH). These indirect-acting mutagens, which require metabolic activation, were produced from combustion of heating oil.

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Ambient-air particulate matter has been estimated at 56×10^{13} g/yr, total global load, including natural pollutants such as dusts, agricultural particulate, and others. The man-made (anthropogenic) global load is estimated to be 28×10^{13} g/yr. Vapor-phase pollutant organics are believed to represent 60 to 90% of the total organic load; the remainder is organics adsorbed onto particulate matter (Hidy and Brock, 1970; Duce, 1978).

Chemical monitoring and analysis of air pollutants have been conducted for many years, but only within the last decade has biotesting technology improved enough to permit simultaneous chemical and biological characterization. However, chemical-analytical expertise exceeds the present biological capabilities, both qualitatively and quantitatively (Hughes et al., 1980).

In this study, air particulate was collected, size-fractionated, solvent-extracted and fractionated into chemical classes, which were then characterized by GC/MS/computer analysis. These chemical class fractions were then biotested for mutagenic activity using the Ames/*Salmonella* bacterial mutagenicity assay. Vapor-phase organic pollutants were also collected and tested. However, no quantitative method was available to adequately measure the mutagenicity of the vapor-phase components; consequently, we are presently developing a bioassay protocol capable of quantifying the mutagenicity of vapor-phase substances. It should be noted that mutagenicity or carcinogenicity is by no means the only significant health hazard that could be presented by air pollutants. As additional *in vitro* bioassay capabilities are developed, other potential toxicity parameters will be examined, such as neurotoxicity and lung toxicity.

METHODS

Collection of Air Particulate and Vapors

Ambient air particulate was collected by the Maxisampler, a high-volume sampling device constructed by the Battelle Corporation after the design by Henry and Mitchel (1978). This device can sample 20,000 m of air in a 24-h period and can collect particulate matter within the respirable range in three size fractions (< 1.7 µm, 1.7 to 3.5 µm, and > 3.5 µm), using impactor plates and electrostatic precipitation. A mechanical diagram of the sampler is shown in Figure 1. After collection, the plates were sealed in a transportable container; and the particulate material was removed and characterized morphologically by scanning electron microscopy. Figure 2 is a typical scanning electron micrograph of ambient air particulate. Air particulate was sampled at five U.S. locations: Elizabeth, N.J.; Upland, CA; Lake Charles, LA; Houston and

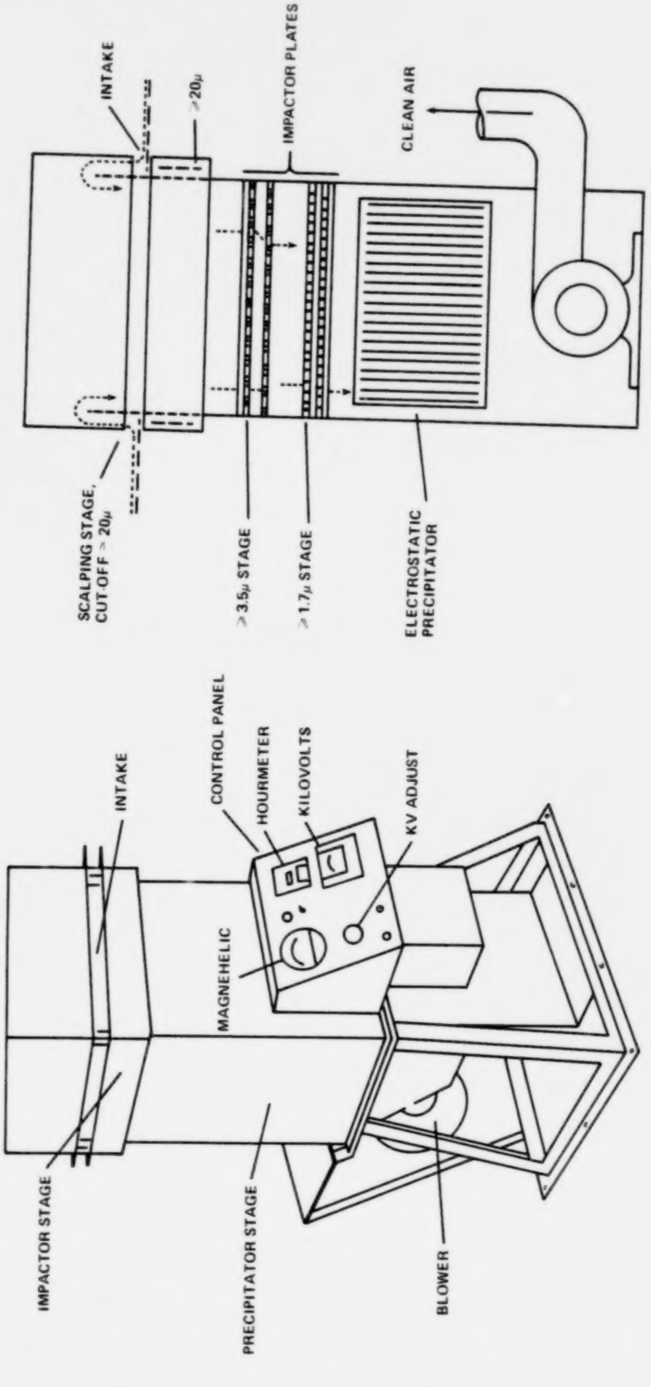

IMPACTOR STAGE
PRECIPITATOR STAGE
BLOWER
INTAKE
MAGNEHELIC
CONTROL PANEL
HOURMETER
KILOVOLTS
KV ADJUST
SCALPING STAGE, CUT OFF > 20μ
≥3.5μ STAGE
≥1.7μ STAGE
ELECTROSTATIC PRECIPITATOR
INTAKE
≥20μ
IMPACTOR PLATES
CLEAN AIR

Figure 1. Schematic of the Battelle massive air volume sampler.

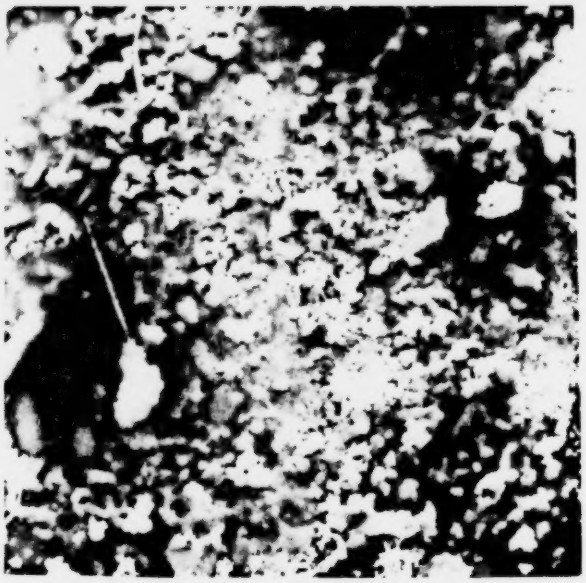


Figure 2. Scanning electron micrograph of ambient air particulate from Lake Charles, LA (> 3.5 μm).

Beaumont, TX. Table 1 summarizes the sampling variables for Elizabeth, N.J.

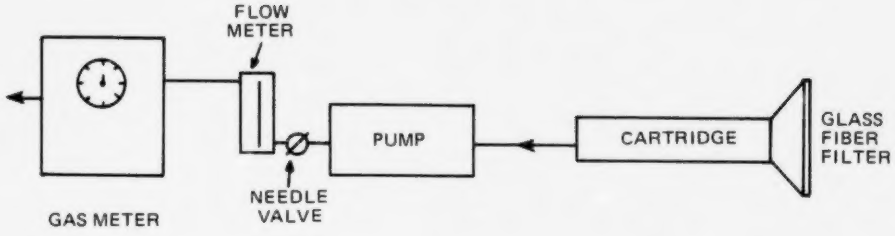
Vapor-phase substances were sampled with cartridges containing Tenax polymeric sorbent. Each cartridge was sealed and later thermally desorbed into cryogenic traps (see Figure 3). This frozen sample was then tested in the Ames/Salmonella assay. Negative results were always obtained, but this may be due to an inadequate testing procedure.

Chemical Fractionation of Ambient Air Particulate

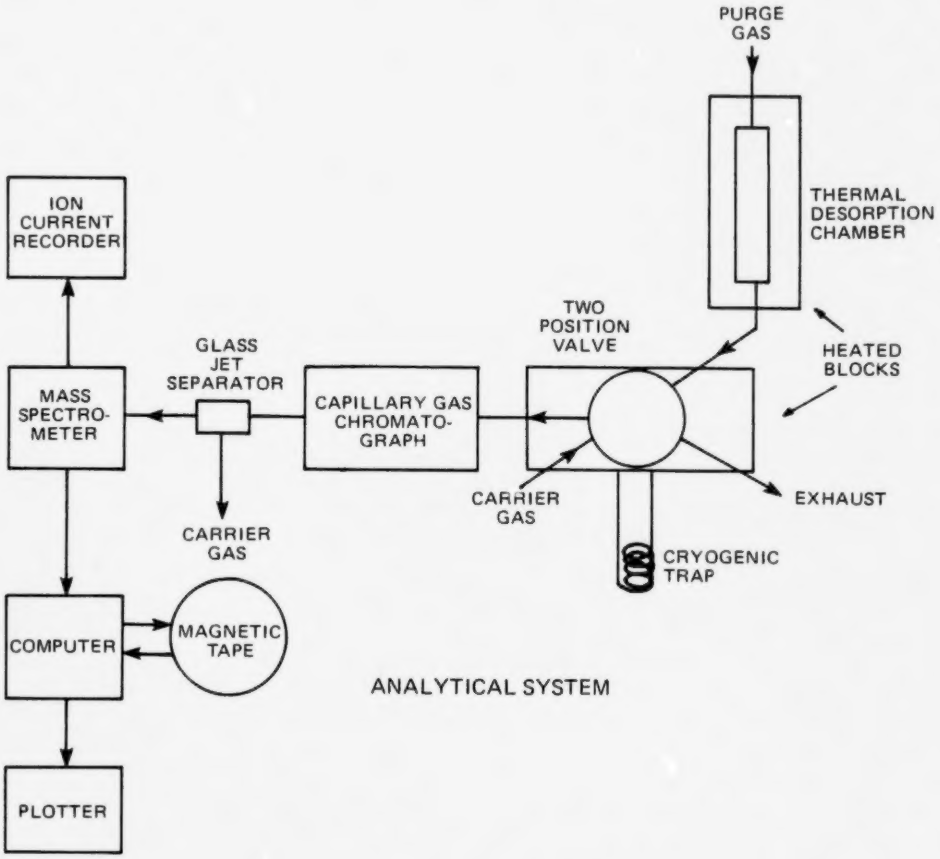
An initial chemical fractionation scheme developed by Pellizzari et al. (1978) that generated 13 polar and nonpolar chemical classes was utilized to chemically fractionate the size-fractionated air particulate. However, the chemical differences among the fractions were small, and dividing the crude particulate into 13 fractions resulted in sample sizes of less than 5 mg, which

Table 1. Sampling Variables for Particulate Organics in Ambient Air in Elizabeth, NJ

Sample Type	Date	Temp. °C (°F)	Sample Vol. (l)	Duration of Sampling (min)	Remarks		
					Time	Relative Humidity	Wind Dir/Vel. kts (m/s)
Vapor-phase organics	9/19-20/78	17 (62)	646	1,390	1515-1425	90%	NE/5-10 kts (2.5-5)
	9/20-21/78	23 (74)	1,211	1,455	1445-1500	91%	SW/10 kts (5)
	9/21-22/78	20 (68)	1,044	1,265	1515-1220	90%	NE/10 kts (5)
	9/22-23/78	20 (68)	1,093	1,335	1245-1100	88%	NE/ 5 kts (2.5)
	9/23-24/78	16 (60)	1,279	1,690	1120-1530	75%	SE/10 kts (5)
Particulates	9/19-22/78		81,211,000	4,560	0805-1200		
	9/22-25/78		78,892,000	4,430	1210-1400		
	9/25-28/78		70,246,000	4,060	1405-0745		


FLOW METER
GAS METER
NEEDLE VALVE
PUMP
CARTRIDGE
GLASS FIBER FILTER

VAPOR COLLECTION SYSTEM


PURGE GAS
THERMAL DESORPTION CHAMBER
ION CURRENT RECORDER
TWO POSITION VALVE
HEATED BLOCKS
GLASS JET SEPARATOR
MASS SPECTRO-METER
CAPILLARY GAS CHROMATO-GRAPH
CARRIER GAS
EXHAUST
CARRIER GAS
CRYOGENIC TRAP
COMPUTER
MAGNETIC TAPE
PLOTTER

ANALYTICAL SYSTEM

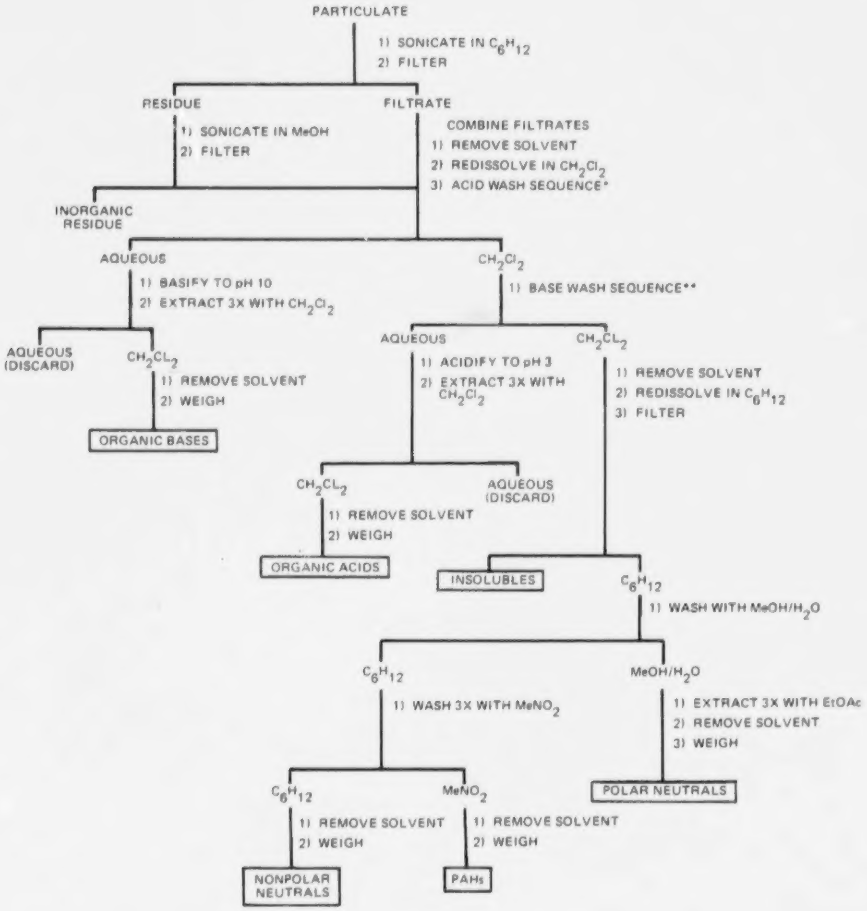
Figure 3. Vapor collection and analytical systems for analysis of organic vapors in ambient air.

limited adequate bioassay and chemical identification. The scheme was modified to generate six chemical classes: acids, bases, PAHs, polar neutrals, nonpolar neutrals, and insolubles (see Figure 4).

Air particulate (~1.0 g) was subjected to ultrasonic treatment in 100 ml cyclohexane (Burdick-Jackson) for 30 min and then filtered through a Teflon (DuPont) filter (0.5-μm pore size). The filtrate was evaporated to dryness using a rotary evaporator; the solids retained by the filter were dissolved in 100 ml methanol (Burdick-Jackson), and sonicated and filtered through the Teflon filter; and the filtrate was taken to dryness. The filtrates were combined, dried, weighed, and redissolved in methylene chloride (CH_2Cl_2). The solution was spiked with internal standards to allow for quantification and to serve as a quality control parameter for the overall partition/analysis scheme. The standards used were quinoline-d7 (organic base), phenol-d5 (organic acid), and anthracene-d10 (PAH).

This CH_2Cl_2 solution was extracted twice with equal volumes each of 10% sulfuric acid (H_2SO_4) and then once with 20% H_2SO_4. The aqueous phases were combined and washed with CH_2Cl_2, and this CH_2Cl_2 phase was combined with the original CH_2Cl_2 solution. The aqueous phase was cooled (ice bath), adjusted with 25% sodium hydroxide (NaOH) to pH 10, and extracted three times with CH_2Cl_2 to generate the "organic bases." The aqueous phase was discarded. The original CH_2Cl_2 solution was extracted three times with 5% NaOH, and the aqueous phases were combined and washed with CH_2Cl_2. The CH_2Cl_2 solution then was combined with the original CH_2Cl_2 solution. The NaOH phases were placed in an ice bath and acidified to pH 3 with 20% H_2SO_4 and extracted three times with CH_2Cl_2 to generate "organic acids." The remaining aqueous phase was discarded.

The original CH_2Cl_2 phase was evaporated to dryness, reconstituted in cyclohexane, and filtered through a Teflon filter (0.5 μm). The cyclohexane filtrate was extracted three times with an equal volume of methanol:water (4:1) solution; the methanol: water extract was then concentrated, and the water extracted three times with ethyl acetate. The solvent was removed to generate "polar neutrals." The cyclohexane phase was extracted three times with an equal volume of nitromethane; the nitromethane phases were then combined and evaporated to dryness to generate the PAHs. The cyclohexane phase was evaporated to dryness to generate the "nonpolar neutrals" fraction.


PARTICULATE
1) SONICATE IN C_6H_{12}
2) FILTER
RESIDUE
FILTRATE
1) SONICATE IN MeOH
2) FILTER
COMBINE FILTRATES
1) REMOVE SOLVENT
2) REDISSOLVE IN CH_2Cl_2
3) ACID WASH SEQUENCE*
INORGANIC RESIDUE
AQUEOUS
CH_2Cl_2
1) BASIFY TO pH 10
2) EXTRACT 3X WITH CH_2Cl_2
1) BASE WASH SEQUENCE**
AQUEOUS (DISCARD)
CH_2CL_2
1) REMOVE SOLVENT
2) WEIGH
ORGANIC BASES
AQUEOUS
CH_2CL_2
1) ACIDIFY TO pH 3
2) EXTRACT 3X WITH CH_2Cl_2
1) REMOVE SOLVENT
2) REDISSOLVE IN C_6H_{12}
3) FILTER
CH_2CL_2
AQUEOUS (DISCARD)
1) REMOVE SOLVENT
2) WEIGH
ORGANIC ACIDS
INSOLUBLES
C_6H_{12}
1) WASH WITH $MeOH/H_2O$
C_6H_{12}
$MeOH/H_2O$
1) WASH 3X WITH $MeNO_2$
1) EXTRACT 3X WITH EtOAc
2) REMOVE SOLVENT
3) WEIGH
C_6H_{12}
$MeNO_2$
POLAR NEUTRALS
1) REMOVE SOLVENT
2) WEIGH
1) REMOVE SOLVENT
2) WEIGH
NONPOLAR NEUTRALS
PAHs

*Acid Wash Sequence: 2 X with 10% H_2SO_4, 1 X with 20% H_2SO_4

**Base Wash Sequence: 3 X with 1 N NaOH.

Figure 4. Fractionation scheme for ambient air particulate organics.

This procedure is a modification of a procedure described by Lee et al. (1976). The partition scheme has been validated for efficiency and presence of spillover of chemicals from one class to another, using mixtures of deuterated known substances representative of the individual chemical classes. Known mutagens were included in the mixture, and the recovery of mutagenic activity before and after fractionation was monitored by the Ames/Salmonella bacterial mutagenicity bioassay.

Mutagenicity Bioassay

The plate-incorporation Ames/Salmonella mutagenicity assay was performed as described by Ames et al. (1975), with and without S-9 rat liver microsomal activation preparation. All assays were conducted with standard mutagens (positive controls) and solvent (negative) controls (samples were exchanged into dimethylsulfoxide [DMSO]). For revertant selection, minimal Vogel-Bonner medium E, supplemented with 1.6% Difco bacto agar and 2% dextrose, was used for base agar layers (25 ml, poured automatically). A 2.5 ml soft agar overlay containing bacteria, minimum amounts of histidine and biotin, and sample was routinely used.

To prepare S-9 from rat liver, the following procedure was used: Male Charles River rats, strain CD_1 (200 ± 20 g), served as the source of liver material. The animals were housed in suspended cages for one week before induction. Food and water were given ad libitum. Induced rat liver (from at least three rats) was obtained from rats injected intraperitoneally on day one with 500 mg/kg of Aroclor 1254 in Mazola Corn Oil (0.5 ml of a 200 mg/ml for a 200 g rat). On day five, the animals were sacrificed and their livers removed. These were immersed in cold, sterile 1.15% KCl, washed two times, and blotted dry with sterile paper. The livers were weighed, minced, brought to 200 mg/ml with 0.25 M sucrose, and homogenized (on ice) with four strokes of a cold Potter-Elvenhjem apparatus with a Teflon pestle. The homogenate was centrifuged for 10 min at 9,000 x g, the lipoprotein layer aspirated off, and the protein concentration of the liver supernatant adjusted (after protein assay) to 30 mg protein/ml with 0.25 M sucrose stock solution. The stock solution was checked for sterility, quick-frozen in small aliquots (2 to 5 ml), and stored for a maximum of two months at -80°C. For the assay, an aliquot of stock solution was slow-thawed and adjusted to an appropriate concentration of protein in 0.25 M sucrose. NADPH was added at 320 µg/plate. Each batch was checked against known positive controls using the plate-incorporation assay. The optimal S-9 protein concentration for mutagenicity was determined by testing with B(a)P and 7-12 dimethylbenzanthracene (DMBA).

Agar diffusion well technique. A modification of the bacterial mutagenicity test was developed to screen for chemical fractions of air particulate organics when sample size was limited. In this test, the sample(s) and S-9 microsomal preparation were placed in wells cut in the agar. Soft agar then was added to fill the well, and after solidification, the bacterial overlay in 2.5 ml soft agar was poured over the entire surface. Results were interpreted as follows: a direct acting mutagen was observed as a ring of colonies around the sample well; a promutagen was observed as a line of colonies between the sample and S-9 wells, and toxicity resulted in a clear zone around the sample well.

This qualitative bacterial mutagenicity test was verified with known mutagens, and its sensitivity was shown to be similar to that of the spot test. Optimum size, number, configuration, and spacing of wells, as well as amount of S-9 rat liver microsomal preparation required per well, were determined. A configuration suited to general screening included a center S-9 well and four surrounding sample and control wells, as illustrated in Figure 5. Advantages of this test over the spot test included ability to 1) measure multiple parameters (dosage, activation requirements, toxicity, and mutagenicity) on a single plate, thereby greatly extending the amount of information obtainable from the available sample; 2) determine positive, negative, and solvent controls on the same plate as the test compound; 3) conduct dose-response and multiple-compound testing on a single plate; 4) eliminate adsorption of test compound by the filter disc; 5) eliminate runoff of liquids onto the agar; and 6) reduce potential loss of mutagenic activity by chemical hydrolysis.

A priority scheme for biological testing was also developed, and was based on total size of each chemical-class fraction:

1) For > 10 mg of sample, the plate-incorporation method was used, with five nontoxic dose levels (at least in duplicate) in the following order: TA98, TA100, TA1535, TA1537, and TA1538. Toxicity was tested at the highest dose only. Preferred initial dose ranges were 1000, 500, 250, 100, and 10 µg/plate. The assay was performed first with, and then without, Aroclor-induced S-9 (3.0 mg/plate).

2) For < 10 mg of sample, the agar well diffusion test was used at two concentrations (500 and 100 µg/plate), except for PNA fractions which were tested using poured plates (500 µg/plate, with Aroclor-induced S-9 and TA98. Duplicate plates were assessed in the following order: TA98, TA1535, TA1537, TA1538, and TA100. Sample was diluted to one concentration for all tests; to obtain lower test concentrations, a smaller sample volume per well was used. Toxicity was tested at 500 µg/plate with TA98 and TA1535, using 1000 colonies/plate.

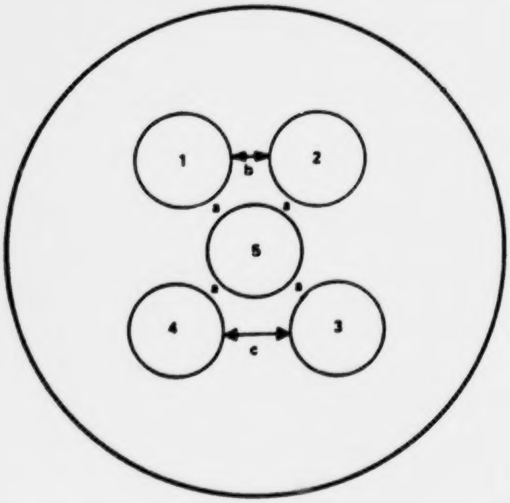


Figure 5. Well testing configuration for bacterial mutagenesis screening. The wells contain: 1) positive control promutagen (e.g., 100 μg of 2-anthramine in 100 μl solvent); 2) DMSO, solvent control (100 μl); 3) low concentration of sample (100 μg sample in 20 μl DMSO); 4) high concentration of sample (500 μg sample in 100 μl DMSO); 5) Aroclor-induced S-9 microsomal preparation ([3.0 mg protein] + NADPH [480 μg in 300 μl]). The distances are: a) 2.5 mm; b) 5.0 mm; c) 10.0 mm. The wells are 18.0 mm in diameter.

RESULTS

Collection, Extraction, and Fractionation

Sampling of 81 x 10^6 l ambient air required 76 h and generated 1.347 g of particulate, of which 180.1 mg were extractable organics, or 2.3 ng organic/l ambient air. Table 2 shows the total particulates collected for each size fraction from each sampling trip. Extractable organics varied from 2.5 to 15.3% of the particulate mass, and the smaller size fractions did not consistently contain the greatest amounts of organics (the 53% extractable organics for Beaumont, TX [< 1.7 μm] is probably incorrect, due to a weighing error). Table 3 illustrates the distribution of the extracted organic after chemical fractionation; 12 to 15% loss of organic matter was common during fractionation.

Table 2. Total Particulate Samples Collected

Sample Location	Particulate Size Range (μm)	Particulate Weight (mg)	Total Organics Extracted (mg)	% Extracted
Upland, CA	<1.7	666	33	5.0
	1.7 to 3.5	641	36	5.6
	>3.5	286	18	6.3
Houston, TX #1[a]	<1.7	337	19	5.6
	>1.7	790	86	10.9
Lake Charles, LA #1	<1.7	747	25	3.3
	>3.5	113	12	10.6
Elizabeth, NJ	<1.7	1347	180	13.4
	1.7 to 3.5	1302	120	9.2
	>3.5	1435	99	6.9
Lake Charles, LA #2	<1.7	2618	401	15.3
	1.7 to 3.5	488	15	3.1
	>3.5	548	27	4.9
Beaumont, TX	<1.7	285	151	53.0
	1.7 to 3.5	882	31	3.5
	>3.5	1701	42	2.5
Houston, TX #2	<1.7	3043	110	3.6
	1.7 to 3.5	1079	–	–
	>3.5	1353	–	–

[a] Particles > 1.7 μm were combined to generate sufficient sample for fractionation and biotesting.

Table 3. Fractionation of Organics Extracted from Ambient Air Particulate Samples

			Amount of Organics in Each Fraction (mg)					
Sample Location	Size Range (µg)	Total Organics Extracted (mg)	Acid	Base	Nonpolar Neutrals	PAHs	Polar Neutrals	% Recovery
Upland, CA	<1.7	32.7	2.9	1.0	6.5	1.6	15.8	85.0
	1.7 to 3.5	17.5	1.8	0.6	4.7	2.0	6.3	88.0
	>3.5	35.9	1.1	0.7	9.4	2.5	16.0	82.7
Houston, TX #1	<1.7	19.3	1.0	0.6	4.0	3.2	9.9	96.9
	>1.7	86.4	0.8	1.2	3.2	2.4	33.2	47.2
Lake Charles, LA #1	<1.7	24.6	7.2	0.1	6.1	2.1	3.7	78.0
	>1.7	11.9	1.7	0.1	3.9	2.3	2.4	87.4
Elizabeth, NJ	<1.7	180.1	8.5	0.5	86.2	11.1	34.6	78.2
	1.7 to 3.5	119.7	10.4	0.4	47.6	9.6	26.1	78.6
	>3.5	99.4	5.5	0.2	52.6	6.0	13.4	78.2
Lake Charles, LA #2	<1.7	401.2	170.2	16.3	49.2	7.1	152.6	98.6
	1.7 to 3.5	15.2	3.9	1.9	4.9	1.5	1.3	88.8
	>3.5	26.8	16.2	3.1	5.3	1.0	0.8	98.5
Beaumont, TX	<1.7	150.6	15.5	6.3	50.3	8.5	6.7	58.0
	1.7 to 3.5	30.7	6.8	0.6	12.2	2.2	8.9	100.0
	>3.5	42.1	10.4	0.4	21.3	4.8	3.8	96.7
Houston, TX #2	<1.7	110.2	20.4	2.8	20.9	12.2	38.0	85.6
	1.7 to 3.5	ND[a]	ND	ND	ND	ND	ND	ND
	>3.5	ND	ND	ND	ND	ND	ND	ND

[a] ND = not determined.

Mutagenicity

Significant mutagenicity was observed for various chemical fractions of the extracted particulate organics from some sites. A sample was considered mutagenic when it generated at least two times the number of revertants over spontaneous background for that bacterial strain. The PAH fraction was mutagenic at all five sites. The polar neutral and organic acids were mutagenic at four of the five sites, and the organic bases at three of the sites. The mutagenicity data on fractions available in quantities too low for the plate incorporation data were determined using the well test. Both indirect- and direct-acting mutagens were present in the complex mixtures and chemical fractions. An example of the mutagenicity data obtained during this study is given in Table 4.

Chemical Analysis

Chemical fractions from each of the organic extracts of the five geographical sites were analyzed by gas chromatography-mass spectrometry (GC/MS). Compounds identified from the analysis were matched against known toxic chemicals from the U.S. Health, Education, and Welfare Chemical Registry and other sources. Table 5 lists known mutagenic, carcinogenic, neoplastic, and teratogenic compounds identified. Many of these toxic compounds were found in the extracted organics from all sites for which chemical analysis was performed. Toxic PAH compounds were best represented.

DISCUSSION

This study was designed to develop and validate an integrated multidisciplinary approach to the study of genotoxic effects of ambient air pollutants. Engineering principles were employed to develop and test the massive air volume sampler used to collect and size-fractionate ambient air particulate. Analytical chemists developed and validated the cyclohexane-methanol extraction scheme for particulate organic matter and the acid-base extractive fractionation scheme to separate the crude organic extract into substituent chemical classes, which were then submitted to the biologist for mutagenesis testing. Such multidisciplinary approaches to the identification of bioactive chemical substituents of various complex environmental mixtures, including organics extracted from ambient air particulate matter, have been employed in recent studies, including those of Epler et al. (1978), Rao et al. (1980), Tokiwa et al. (1977), and others.

Table 4. Mutagenesis Data of Particulate Fractions from Lake Charles, LA (<1.7 μm)[a]

Sample Fraction	Doses (μg/plate)	MA Protein Concentration (mg)	TA100		TA98		TA1538		TA1537	
			+MA[b]	-MA	+MA[b]	-MA	+MA[b]	-MA	+MA[b]	-MA
Total organics	1000	3.0	507* ± 17	450* ± 21	127* ± 28	210* ± 70	152* ± 28	125* ± 12		
	500	3.0	327 ± 15	341 ± 30	74 ± 24	169* ± 12	60 ± 5	80 ± 0		
Polar neutrals[c]	500	3.0	401 ± 18	387 ± 2	129* ± 51	241* ± 6	117 ± 6	191* ± 12	67 ± 2	21 ± 11
	100	3.0	186 ± 63	256 ± 73	94 ± 23	155* ± 9	56 ± 8	74 ± 12	24 ± 10	23 ± 9
Acids[c]	500	3.0	326 ± 9	221 ± 91	82 ± 15	77 ± 17	59 ± 21	67 ± 11	62 ± 17	19 ± 10
	100	3.0	150 ± 95	168 ± 57	63 ± 20	89 ± 22	61 ± 5	52 ± 12	47 ± 21	39 ± 4
Bases	500	1.5	163 ± 106	94 ± 63	105* ± 17	51 ± 16	69 ± 1	51 ± 8		
	100	1.5	197 ± 71	276 ± 25	77 ± 21	66 ± 19	56 ± 3	46 ± 0		
	500	3.0			103* ± 40					
	100	3.0			53 ± 24					
	500	6.0			58 ± 19					
	100	6.0			62 ± 17					
Nonpolar neutrals	500	3.0	398 ± 13	224 ± 21	122* ± 7	107* ± 45	69 ± 5	53 ± 3		
	100	3.0	365 ± 7	279 ± 25	93 ± 9	88 ± 10	60 ± 7	37 ± 0		
Polynuclear aromatic hydrocarbons	500	1.5			1007* ± 159					
	100	1.5			394* ± 53					
	500	3.0			755* ± 144					
	100	3.0			283* ± 58					
	500	6.0			257* ± 198					
Positive control	100		2404* ± 218	280 ± 13	2309* ± 12	1848* ± 41	2086* ± 5	1260* ± 55	39 ± 20	174* ± 38
	10		2197* ± 682	1241* ± 215	2074* ± 31	1117* ± 47	1529* ± 13	623* ± 27	69 ± 20	20 ± 6
	1		1061* ± 14	627* ± 177	635* ± 38	109* ± 17	683* ± 30	339* ± 78	74 ± 6	20 ± 2
Solvent control	0.1 ml		218 ± 16	205 ± 9	51 ± 3	53 ± 5	64 ± 5	44 ± 3	38 ± 6	24 ± 6

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[a] Plate-incorporation assays; results reported in mean ± standard deviation computed with triplicate plates.

[b] MA = metabolic activation.

[c] The polar neutrals and acids were tested in TA1535; however, the spontaneous revertants were not within acceptable limits.

*Mutagenic response is at least twofold that of the spontaneous revertants.

Table 5. Mutagenic, Carcinogenic, Neoplastic, and Teratogenic Compounds[a] Identified in Ambient Air

Compounds	Upland CA	Lake Charles LA (1)	Lake Charles LA (2)	Elizabeth, NJ	Beaumont, TX	Houston TX (1)	Houston TX (2)	Mutagenic	Carcinogenic	Neoplastic	Teratogenic
Acenaphthene				+						+	
Allyl-phenol	+									+	
Amino-anthracene	+							+		+	
Amino-anthraquinone	+					+				+	
Aniline		+							Indefinite		
Anthanthrene				+					+	+	
Anthracene	+	+	+	+		+			+	+	
Anthraquinone	+	+								+	
Benz-anthracene	+	+	+	+		+		+	+		
Benzo-fluoranthene		+	+	+		+			+	+	
Benzo-perylene			+	+					+	+	
Benzo-phenanthrene	+	+	+	+		+			+		
Benzo-pyrene	+	+	+	+		+		+	+		
Bromophenol		+								+	
Butylbenzylnitrosamine						+		+	+		+
Caffeine						+					+
Chlorophenol	+										+
Chrysene	+	+	+	+		+		+	±		
Cresol	+					+				+	
Dicyclohexylamine		+								+	
Dimethyl-anthracene						+				+	
Dimethyl-phenanthrene	+			+						+	
Fluoranthene	+		+	+		+				+	
Methylbenz-anthracene	+	+		+				+	+	+	
Methylbenz-phenanthrene	+	+	+	+				+	+	+	

[a] U.S. Department of Health, Education, and Welfare, 1977.

(continued)

Table 5. Mutagenic, Carcinogenic, Neoplastic, and Teratogenic Compounds[a] Identified in Ambient Air (continued)

Compounds	Upland CA	Lake Charles LA (1)	Lake Charles LA (2)	Elizabeth, NJ	Beaumont, TX	Houston TX (1)	Houston TX (2)	Muta-genic	Carcino-genic	Neo-plastic	Terato-genic
Methyl-chrysene		+		+						+	
Methylene-phenanthrene	+									+	
Methyl-phenanthrene	+	+		+		+				+	
Methyl-pyrene	+	+		+		+				+	
Methyl-stearate	+		+	+		+				+	
Naphthacene	+	+							+		
Naphthalene	+	+				+				+	
Perylene	+	+	+	+		+			+		
Phenanthrene	+	+	+	+		+				+	
Phenol	+	+				+			+		
Phenyl-benzene				+						+	
Phenylene-pyrene				+					+		
Phenyl-β-naphythlamine	+			+		+		+	+	+	
Phenylphenol		+								+	
Progesterone				+					Suspected	+	+
Propenyl-phenol	+									+	
Pyrene	+	+	+	+						+	
Quinoline	+							+	+		
Styrene	+							±	±		
(p-Tetramethyl-butyl)phenol		+								+	
Tetrahydrobenzo-phenanthridine						+				+	
Toluidine						+			+	+	
Trimethyl-phenanthrene				+						+	
Triphenylethylene						+				+	

[a]U.S. Department of Health, Education, and Welfare, 1977.

Several problems were encountered in the development and validation of this technical approach to the investigation of the bioactivity of air particulate matter. Technical considerations concerning the specificity of the chemical fractionation scheme have been discussed previously (Pellizzari et al., 1978). The chemical fractionation scheme separates organic acids and bases by treatment with aqueous base and aqueous acid, followed by partitioning into organic solvent. Löfroth (1980) contends that such treatment may generate artifacts, especially in the organic base (nitrogen-containing compounds), due to reaction of substances with the aqueous acid or base during the extraction. Epler et al. (1978; Rao et al., 1980) recommended separation of the acid and base components with Sephadex LH-20 to avoid this problem; however, they found no significantly different results using LH-20 vs. acid-base extraction.

Sample size was a chronic problem in this study. The massive air volume sampler did not collect the amounts of particulate expected. In a 10-day sampling period, often less than 3 g particulate matter, including all three size fractions, was collected. Only 5 to 15% of the particulate represents extractable organics, and this material, when partitioned between five fractions, usually did not provide enough sample for quantitative dose-responsive plate-incorporation mutagenicity assays. For triplicate determinations in all five Salmonella strains, using five doses, both with and without metabolic activation (as suggested by deSerres and Shelby, 1979), the assay would require 50 mg. Enough material must also be available for chemical analysis. As can be seen from Table 3, none of the samples were of adequate size for complete bioassay and chemical analysis.

Despite the limited data available, the results of air particulate research by various investigators at different sites show certain similarities to this study. For example, Pitts et al. (1977) collected air particulate at rural and urban sites in California and extracted the organics using sonication with equal parts of methanol, benzene, and dichloromethane. The particulate collected from the rural site was not mutagenic, but material from all urban locations was mutagenic without S-9 addition, exhibiting a linear dose response for Salmonella strains TA98, TA1538, and TA1537. Thus, the mutagenic components present were probably not polynuclear aromatic hydrocarbons (PAH), which require activation. In another case, mutagenic activity requiring S-9 metabolic activation was demonstrated in a California air sample; this activity was then quantitatively related to B(a)P concentration (Flessel, 1980).

In Japan, ambient air was sampled at six residential and industrial sites (Tokiwa et al., 1977). The organic fraction from ambient air particulate (extracted with methanol) collected in

Ohmuta (an industrial city) was mutagenic, exhibiting a linear dose response from 100 to 400 μg with Salmonella strain TA98. A sample collected from a residential area (Fukuoka City) required from 370 to 1970 μg/plate to generate a positive mutagenic response. The mutagenicity was approximately three times greater for the sample collected from the industrial city, and a linear dose response was observed for both samples. These researchers identified the compounds in the methanol extract using an alumina column and GC/MS analysis. A majority of the compounds identified were PAHs such as benzopyrenes, anthracenes, fluorenes, and dibenzoisomers of these compounds. These Japanese studies revealed that air particulate near industrial sites possessed higher mutagenic potential than that from rural sites and that both S-9-activated (PAH) and direct-acting (non-PAH) mutagenic components were present in air particulates, agreeing with the California studies of Pitts et al. (1977) and Flessel (1977).

Talcott and Wei (1977) collected air particulate on glass fiber filters and extracted the filter with acetone in a Soxhlet apparatus. The extracted material exhibited the highest activity with TA100 and required S-9 metabolic activation. Direct-acting mutagens were also detected with TA98 and TA1537. Activity was again attributed to at least two types of mutagens: a PAH and a non-PAH fraction. The view that PAH compounds are not the sole (or major) mutagenic factor associated with air particulate was further reinforced by the studies of Dehnen et al. (1977). Here, mutagenic activity was predominantly produced by compounds other than PAHs, since the observed mutagenicity did not require S-9. Dehnen used a chemical fractionation scheme to divide his crude air particulate sample into cyclohexane and methanol-extractable fractions. An alumina column was employed to further fractionate the cyclohexane extract into a purified cyclohexane fraction (containing PAH-type compounds) and a 2-propanol fraction (containing azo-heterocyclic compounds). The highest mutagenic response was found in the 2-propanol and methanol extracts; neither fraction contained PAH-like compounds.

Other investigators (Tokiwa et al., 1977) have combined a chemical fractionation scheme with a mutagenesis-detection system. After solvent extraction, fractionation of the crude organic extract into chemical classes permitted identification of the mutagenically active chemical compounds within each chemical class. In some cases, fractionation appeared to reduce or remove toxic effects that otherwise would have prevented expression of mutagenicity. Thus, the mechanism of synergism or antagonism of the individual components in the crude mixture is open to investigation. Pelroy et al. (1978) observed such effects with shale oil and its fractions, as did Rao et al. (1980) with fossil fuels and their fractions.

Pellizzari et al. (1978) initially tested a West Virginia ambient air particulate for mutagenicity using a fractionation scheme and the qualitative spot test (Ames et al., 1975) with the standard Ames Salmonella tester strains (TA98, TA100, TA1535, TA1537, and TA1538), both with and without a metabolic activation system (S-9). Mutagenic activity was observed for the organic bases, organic acids, and aromatics. Each of the five tester strains gave a positive response with at least one of the active fractions, and only the aromatics required metabolic activation (Hughes et al., 1978; Pellizzari et al., 1978). In this West Virginia sample, 2-nitro-4,5-dichlorophenol, a direct-acting mutagen (Pellizzari et al., 1978), was identified in the polar neutrals, and fluoranthene, pyrene, benz(a)anthracene, and B(a)P were identified in the aromatic fraction. All of these compounds have been identified either as co-carcinogens (Van Duuren, 1976) or as mutagens requiring metabolic activation (McCann et al., 1975).

These preliminary results indicated that the Ames assay could detect mutagenic activity in small sample amounts of ambient air (as a complex mixture), and that the fractionation and analysis scheme was capable of identifying broad chemical mutagenic classes within these mixtures. It was concluded that chemical fractionation of the crude complex sample was both useful and necessary to accomplish adequate biotesting and chemical identification of signature mutagenic components in ambient air particulate.

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EVALUATION OF COLLECTION AND EXTRACTION METHODS FOR MUTAGENESIS STUDIES ON AMBIENT AIR PARTICULATE

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INTRODUCTION

The extractable organics associated with air particles have been shown to be carcinogenic (Hueper et al., 1962; Leiter et al., 1942) and mutagenic in a short-term microbial bioassay (Lewtas Huisingh, 1980; Teranishi et al., 1977). The identification and characterization of the potentially hazardous chemical components associated with ambient air particles requires that efficient collection and extraction methods be developed and validated. It is important that the original chemical composition of the particle-bound organics be maintained through collection and extraction to establish that the bioassay results are directly relatable to the chemistry of the original organics as they exist on particles in the atmosphere. The particles of major interest are those that are in the inhalable (< 15 µm) or respirable (< 5 µm) size range and therefore can be trapped in the human respiratory tract. Several studies have shown that the organics associated with ambient air particles are more mutagenic in the smaller (respirable) particle size range (Huisingh, 1980; Teranashi et al., 1977).

In the past, most conventional ambient air samplers were not designed to collect particles in size-separated stages, nor could they collect the quantities of particulate matter desired for integrated biological and chemical studies. A new sample collection instrument, the Massive Air Volume Sampler (MAVS), has been designed to separate collected particles into three size ranges (0 to 1.7 µm, 1.7 to 3.5 µm, and 3.5 to 20 µm) and to collect large amounts of particles. The objective of this study is

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to evaluate current ambient air particle sampling and extraction methods for short-term mutagenesis bioassay applications.

COMPARISON OF AVAILABLE COLLECTION METHODS

A great number of field air particle samplers with varied applications have been designed and used. Filtration and impaction are the two techniques most commonly employed to collect particles for the determination of particle concentration and chemical composition. The conventional air particle collection instrument, recommended in the Federal Register for determining total suspended particulate (TSP), is the standard high-volume sampler. Recently, the size selective inlet (SSI) high-volume sampler has been introduced to collect size-selected inhalable particles less than 15 µm (McFarland and Rodes, 1979). In addition, the dichotomous sampler (virtual impactor) is being employed when elemental chemical analysis is desired (McFarland and Rodes, 1979). All three of these instruments employ filtration as the primary collection method. Preliminary studies have shown that these samplers do not collect sufficient amounts of particles for complete bioassay studies. Henry and Mitchell (1978) designed and constructed a massive air volume sampler (MAVS) for the U.S. Environmental Protection Agency (EPA) that would collect large quantities of size-fractionated air particles. The principles and operation of this sampler are described in the next section. The new sampler, MAVS, capable of collecting gram amounts of sized particles in a reasonable sampling period, is currently being evaluated for collecting ambient air particles to be used in bioassay studies.

A comparison of particle mass that can theoretically be collected by the TSP high-volume sampler, the SSI high-volume sampler, the dichotomous sampler, and the new MAVS is shown in Table 1 for different air pollution TSP concentrations. The comparison was made for 24-h sampling periods in ambient air with TSP particle concentrations of 60, 100, and 200 µg/m^3. As noted in the table, the mass of particles collected depends on the ambient particle concentration and, more importantly, on the flow rate of sample air through the given sampler. The flow rate of the MAVS (18.5 m^3/min) enables it to collect a greater amount of particulate matter than the other samplers.

The Massive Air Volume Sampler

Since the MAVS is a relatively new particle sampler, a brief description of its operation is given here. The sampler, shown in Figures 1, 2, and 3, has an inlet which serves as the scalping stage that permits only particles of 20 µm aerodynamic diameter or

Table 1. Comparison of Amounts of Particulate Matter Theoretically Collected by Four Types of Samplers

Type of Sampler	Flow Rate (m^3/min)	Vol. of Air Sampled[a] (m^3)	Calculated Particulate Matter Collected at: TSP Concentrations[a] 60 µg/m^3 (g)	100 µg/m^3 (g)	200 µg/m^3 (g)
Dichotomous	0.017	24.0	0.0015	0.0024	0.0048
SSI high-volume	1.13	1,627.0	0.097	0.16	0.33
TSP high-volume	1.40	2,016.0	0.12	0.20	0.40
MAVS	18.5	26,640.0	1.6	2.7	5.3

[a] During 24-h sampling period.

less to enter the sampler. The sample air then enters an impaction plate assembly containing four stainless steel Teflon-coated plates with slots, which serve as impactor jets and collection surfaces for the two-stage impactor. The first stage of the impactor collects particles between 20 and 3.5 µm, and the second stage collects particles between 3.5 µm and 1.7 µm. Immediately below the impaction plates, the remaining particles less than 1.7 µm pass through a particle charging field and are collected on the third stage, which consists of vertically charged electrostatic precipitator (ESP) plates. Both the impaction plates and the electrostatic collector plates are Teflon-coated to minimize substrate contamination and to facilitate removal of the particles. The particle collector plate assemblies, after sampling, are shipped in sealed containers to the laboratory, where the particles are mechanically scraped from the Teflon plates. Figure 4 shows the particles being removed inside a sealed glove box to minimize contamination of the sample and to insure the safety of personnel.

Ozone Generation in the MAVS

The MAVS does not have the potential artifact-formation problems associated with substantial flow of reactive gases (e.g., ozone and nitrogen oxides) over particles collected on reactive filtration media, since the MAVS employs impaction and

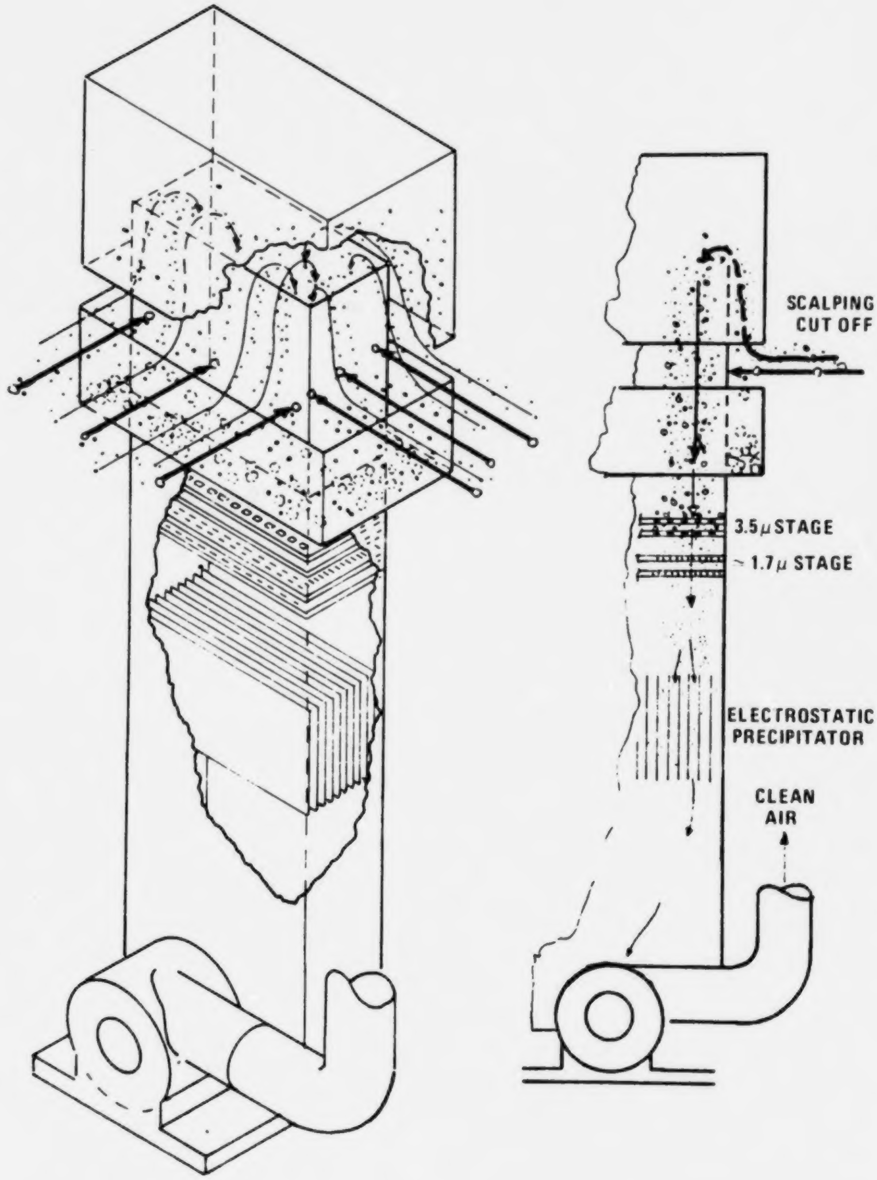
SCALPING STAGE,
CUT OFF ~ 20 μ
3.5 μ STAGE
~ 1.7 μ STAGE
ELECTROSTATIC
PRECIPITATOR
CLEAN
AIR

Figure 1. Massive Air Volume Sampler schematic.

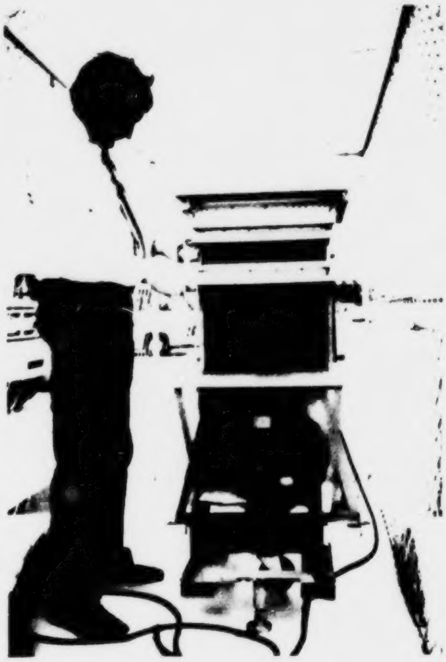

Figure 2. Massive Air Volume Sampler.

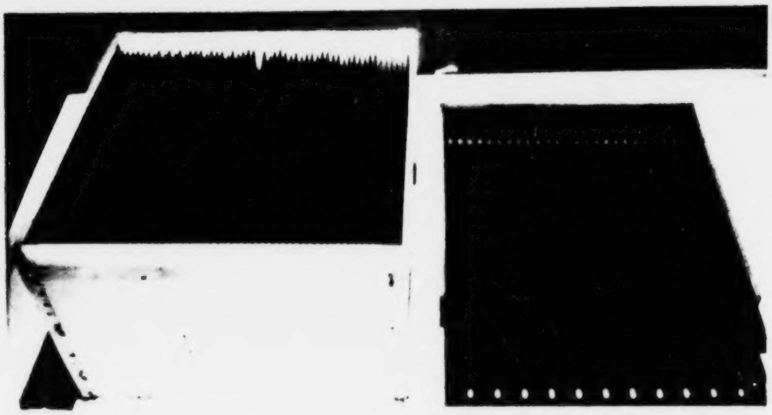

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Figure 3. Impactor plate and electrostatic precipitator.

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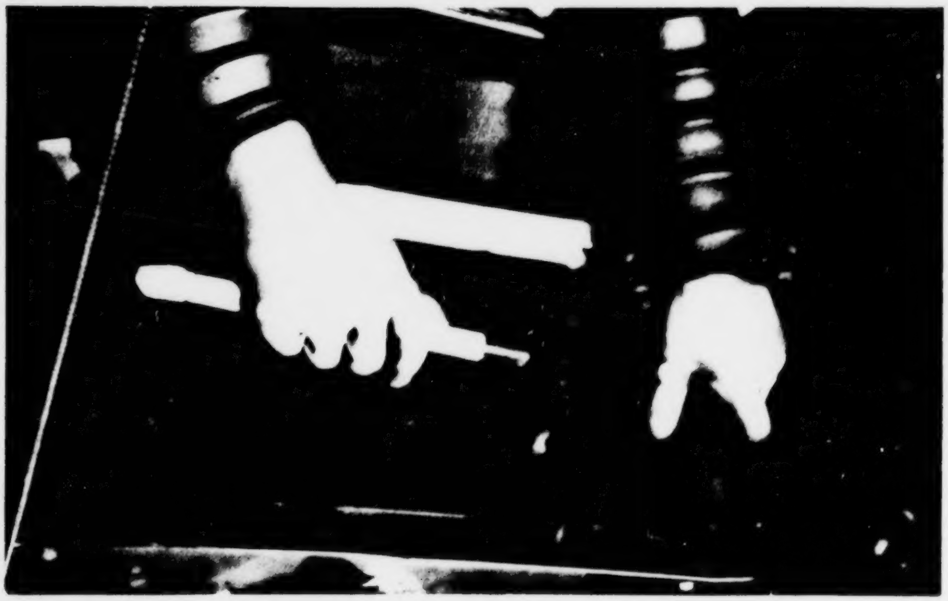

Figure 4. MAVS plate being scraped inside glove box.

electrostatic precipitation onto Teflon-coated surfaces. The ESP particle-charging section of the sampler, however, generates low levels of ozone that passes through the fine particle (< 1.7 µm) collection stage. Since ozone may react with organic compounds (NSF, 1977), including polynuclear aromatic hydrocarbons (PAH), it is important to determine whether the generated ozone reacts with the organic compounds associated with the collected particles and whether such reactivity would bias mutagenicity bioassay results.

Mitchell et al. (1978) reported the ozone concentrations present in the blower outlet of the first MAVS under different operating conditions. After subtracting the ambient background ozone (0.05 ppm), they reported no ozone emitted when a positive corona was employed and 0.025 to 0.050 ppm ozone emitted when a negative corona was employed. As the corona sign was changed from positive to negative, the particle collection efficiency increased from 66% to 80% at a corona voltage of 7,800. As the negative corona voltage was increased, both collection efficiency and ozone concentration increased.

As the MAVS has become viable as the most acceptable method for collecting large samples of size-separated ambient particles for bioassay screening studies, artifact formation due to reaction of the collected material with ozone has become an important consideration. Extractable organics from ambient air particles collected at the third stage of the MAVS, the ESP, where an ozone reaction would be most suspect, were found to be more mutagenic than the larger particles collected at the first and second stages (Huisingh, 1980). A second, more detailed measurement of ozone within the sampler, as well as at the inlet and outlet, was performed; the results are given in Table 2. The standard operating particle charging voltage of 10,000 volts was used along with negative polarity and corona. As noted in Table 2, the highest ozone concentration in this study was found at the blower outlet of the sampler, which is downstream of the third collection stage. The highest ozone concentration observed in any section of the sampler where particles are actually collected was 0.08 ppm. When the voltage was reversed to give a positive corona, no measurable amounts of ozone were generated in the sampler.

Table 2. MAVS Ozone Generation Test Results

Charging Wire Voltage[a]	Plate Voltage[a]	Location	Ozone (O_3) Generated (ppm)
10,000	7,800	Sampler inlet	---[b]
10,000	7,800	Precipitator inlet	0.07
10,000	7,800	Between plates	0.08
10,000	7,800	Precipitator outlet	0.09
10,000	7,800	Blower outlet	0.11
10,000	5,000	Between plates	0.08

[a] All voltages are negative polarity.

[b] Room ambient concentration was 0.02 ppm O_3. All other values were corrected for this amount to determine the concentration of O_3 generated.

MAVS Ozone Field Study

A field study was developed to determine whether the concentration of ozone generated in the electrostatic precipitator collector section of the MAVS causes reaction between the ozone and the organic constituents of the collected particles and consequently biases the results of the bioassay. Three different sampling sites were selected on the basis of possible PAH presence in the air: Durham, NC (EPA Cameo Building in downtown Durham); Birmingham, AL (industrial site in north Birmingham); and Gadsden, AL (steel mill coke oven).

Since the MAVS can be operated with either positive particle charging corona (without ozone generation) or with negative particle charging corona (with ozone generation), field samples were collected by co-located samplers (one with positive charging corona and one with negative charging corona). All samples were extracted, and split blind samples were both chemically characterized and tested in the Ames *Salmonella typhimurium* microbial mutagenesis bioassay. Chemical characterization and bioassay results from particles charged with positive corona were compared with results from particles charged with negative corona. Samplers were operated in a normal manner with particle charging voltage of 10,000 volts and collector plate voltage of approximately 7,800 volts. As shown in Table 2, 0.08 ppm of ozone was generated in the negative charging mode and no measureable ozone in the positive charging mode.

At the Birmingham site, a sample was collected simultaneously by each of the two co-located samplers (one with positive and one with negative particle charging corona). The same sampling scheme was used in Gadsden, where the two co-located samplers were operated for two sequential sampling periods. In Durham, the same sampling scheme was used, with the sampler corona reversed in the second of the two sequential sampling periods to eliminate any possible sampler bias. The sampling scheme for all three tests is shown in Table 3.

The particles collected at the Durham field site were mechanically extracted from the collector plates, and the Birmingham and Gadsden particles were rinsed from the collector plates with dichloromethane (DCM; methylene chloride). These particle samples and solvent extract samples were sealed and blind-coded for laboratory analysis (e.g., organic extraction, chemical analysis, and bioassay preparation).

Table 3. Ozone Field Study Sampling Scheme

		Particle Charging Corona	
Location	Sampler	Experiment 1	Experiment 2
Durham, NC	A	+	-
	B	-	+
Birmingham, AL	A	+	
	B	-	
Gadsden, AL	A	+	+
	B	-	-

Analysis Methodology

The air particle samples from Durham were Soxhlet-extracted with DCM after mechanical removal from the plates, and those from Birmingham and Gadsden, which were originally received in DCM, were filtered. The DCM extract from both types of samples was dried in a stream of dry nitrogen. One aliquot was used for chemical analysis, and one aliquot was prepared for bioassay by adding dimethylsulfoxide (DMSO) to obtain a concentration of 2 mg/ml.

The DCM extracts were prepared for gas chromatographic (GC) and GC/MS analysis of polycyclic aromatic hydrocarbon (PAH) using procedures developed by Bjϕrseth (1977). The GC analysis was performed on these extracts with a flame ionization detector (FID) and a glass capillary column with hydrogen as the carrier gas. Splitless injections were used while the column was at ambient temperature, and peak area integration was performed with an automatic digital integrator.

The GC/MS analysis was performed using a Finnigan Model 3200 MS and a Model 9500 GC with a glass capillary column. Splitless injections were made while the column remained at ambient temperatures. Electron impact (EI) spectra were obtained and processed with a Digital System 150 and INCOS data system.

Bioassay Methodologies

The *S. typhimurium* plate incorporation assay was performed as described by Ames et al. (1975), with minor modifications. The modifications consisted of adding the standard minimal concentration of histidine directly to the plate media instead of to the overlay and then incubating the plates for 72 rather than 48 h. The comparative samples were bioassayed simultaneously in the same experiment for each tester strain.

The studies were performed with triplicate plates at five doses with and without metabolic activation. Activation was provided by a 9000 x g supernatant of liver from Aroclor-1254-induced CD rats (Ames et al., 1975). The linear portion of the dose-response curves were used to calculate a linear regression line. The slope of the linear regression analysis is reported as revertants per microgram (rev/μg) of organics tested.

Discussion of Analytical Results

GC chromatographs were obtained under identical conditions and show a similar relative concentration of PAH components in the pairs of samples for all three sites. This similarity was shown regardless of collection date, precipitator ion potential, or whether the precipitator plates were washed or scraped.

The similarity noted in the chromatograms was the basis of a reduced number of samples being analyzed by GC/MS. With the identification of the major components determined by GC/MS, the total ion chromatogram was compared to the GC chromatograph to aid in the identification of the various components. The quantification for various PAH species and the concentration ratios for two pairs of isomeric PAH are shown in Table 4.

Some PAHs are oxidized readily, while others are relatively insensitive to oxidation (Committee on Biological Effects, 1972). Pyrene and anthracene are readily oxidized, while fluoranthene and phenanthrene are relatively inert to oxidation. Assuming that reactions with ozone proceed in the same manner in the MAVS with negative corona as in the laboratory studies, the ratio between the inert and reactive compounds should reflect any potential reactions of ozone with the reactive PAHs. Comparison of these ratios (Table 4) in the presence and absence of ozone (negative and positive corona) show no significant or consistent changes, suggesting no significant reaction of the PAHs with ozone. In fact, the values obtained were well within the range reported in other studies, including ambient air and aluminum and coke plants that were conventionally sampled (Bjϕrseth et al., 1978; Hoffman and Wynder, 1976).

Table 4. Comparisons of PAH Levels at Three Sampling Locations[a]

Chemical Compound	PAH (μg/g)					
	Durham		Birmingham		Gadsden	
	+	-	+	-	+	-
Phenanthrene	1470	872	116	64	776 896	616 940
Anthracene	1960	1150	92	72	288 256	180 524
Ratio P/A	0.75	0.76	1.23	0.90	2.69 3.46	3.46 1.79
Fluoranthene	2190	648	208	196	924 1840	1370 2220
Pyrene	1980	696	224	184	1250 1900	1240 2900
Ratio F/P	1.11	0.93	0.94	1.08	0.74 0.97	1.10 0.77

[a] Positive corona = +; negative corona = -.

The data provide interesting information about the MAVS. Since the Birmingham samples (#1 and #2) were collected in the same manner, they should exhibit similar patterns. In order to study this relationship, parent PAH profiles (PPP) were constructed. PPP depict relative distribution of the key PAH compounds in the sample, thus providing a convenient method of comparing samples (see Figure 5). The profile remained relatively constant over the sampling period, indicating that operation of the electrostatic precipitator in the positive corona (#1) or negative corona (#2) mode did not affect the composition of the samples.

The data indicate that no significant degradation of PAH attributable to ozonation occurred, compared with the inert compounds. The stability of the profiles indicates that variation of sampling parameters had no influence on the collected organics. Therefore, it is unlikely that the MAVS electrostatic precipitation

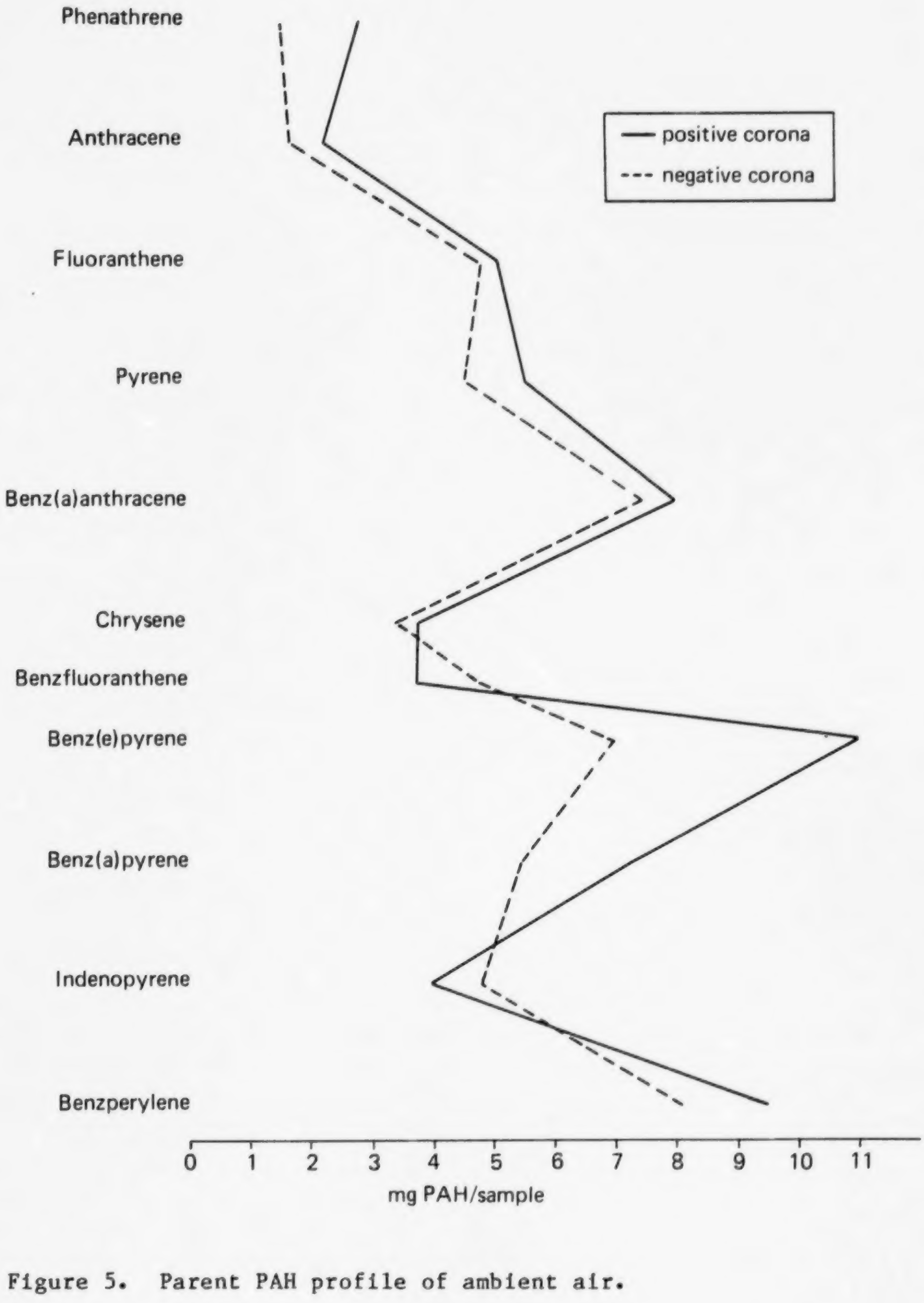

Figure 5. Parent PAH profile of ambient air.

plates in the positive or negative corona mode caused any artifact formation due to ozonation.

Discussion of Bioassay Results

The mutagenic activity in S. typhimurium tester strain TA98 is shown in Figure 6 for each of the three sites as a function of ionization potential. At the Durham site, the slope of the mutagenic activity curve ranged from 0.37 to 0.42 rev/µg in the absence of S-9 activation and from 0.52 to 0.75 rev/µg in the presence of activation. No significant difference was observed in mutagenicity as a function either of ionization potential or of sampler.

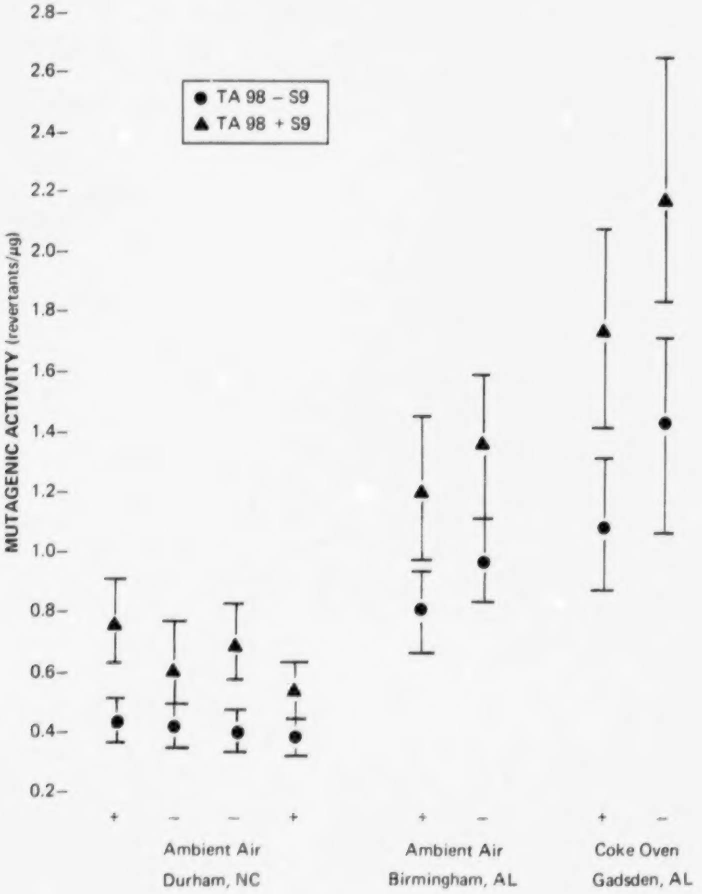

Figure 6. Corona potential.

Although the mutagenic activity of the Birmingham and Gadsden samples was greater than the Durham samples, no significant difference was observed between the two samples collected during the same time periods with different coronas.

Evaluation of Extraction Techniques

To determine which extraction technique was the most effective in removing mutagens from ambient air particles, two extraction methods and seven solvent systems were evaluated. The two extraction methods were Soxhlet extraction for 24 h and sonication for both 30 min and 2 h. The four basic single solvents, selected for increasing polarity index, were cyclohexane (CH), dichloromethane (DCM), acetone (Ac), and methanol (MeOH), with polarity indices of 0.0, 3.4, 5.4, and 6.6, respectively. Three additional comparative solvent systems were sequential sonication of cyclohexane and methanol (CH/MeOH), 1:1:1 toluene: dichloromethane:methanol (T:DCM:MeOH), and direct suspension in dimethylsulfoxide (DMSO). These solvent systems were selected for comparison with previous work (Pitts et al., 1977; Pellizzari et al., 1979). Only the four single solvents were used in the Soxhlet extraction method, while two of the comparative solvent systems were also used in sonication.

The data in Table 5 indicate no significant difference for percent extractables in the length of time used for sonication, but acetone and methanol extracted approximately three times as much mass as cyclohexane and twice as much as DCM. The Soxhlet extraction method indicated a significant increase of percent extractables using acetone as the solvent.

Table 5. Ambient Air Particles Percent Extractables

Solvent	Soxhlet 24 h	Sonication 30 min	Sonication 2 h
CH	4.0	2.9	3.0
DCM	6.4	4.3	4.7
Ac	21.0	9.0	8.2
MeOH	8.9	11.4	11.5
CH/MeOH		11.6	11.6
T:DCM:MeOH		8.7	8.9

An aliquot of each extract was dried under nitrogen and diluted with a 1:1 mixture of DCM and cyclohexane. Fifty microliters were spotted on a TLC plate and developed in a tank containing a 1:2 mixture of DCM and ethanol. Analysis was done with a Perkin Elmer MPF-44B Fluorescence Spectrometer. The results are shown in Table 6. Regardless of method of extraction or solvent used, the benzo(a)pyrene (B[a]P) analysis is relatively constant on a weight-by-weight basis.

Table 6. Ambient Air--Glass Fiber Filters B(a)P Analysis

	B(a)P (μg/g)		
Solvent	Soxhlet 24 h	Sonication 30 min	Sonication 2 h
CH	11.8	8.2	8.4
DCM	12.0	10.9	9.5
Ac	11.4	11.7	10.1
MeOH	11.8	11.1	10.4
CH/MeOH		13.2	12.8
T:DCM:MeOH		13.9	11.7

An aliquot of 10% of the extract was dried and prepared in aqueous solution for analysis by a Dionex Model 10 ion chromatograph (IC). The IC was equipped with a standard column and 0.003 M sodium bicarbonate and 0.0024 M sodium carbonate eluent. The sample was analyzed for fluoride, chloride, nitrate, and sulfate ions. Total anion concentration was calculated and compared for the four single solvents used in Soxhlet extraction and the two additional comparative solvent systems used in the sonication extraction techniques. Table 7 shows that the increasing polarity of the solvent is comparable to the increasing quantity of total anions extracted, regardless of extraction technique, although the increased time of sonication did extract increased amounts of total anions. Table 7 also shows that the methanol in both the Soxhlet and 30-min sonication extraction techniques and the comparative sorbent system CH/MeOH in the 30-min sonication extraction technique extracted approximately the same quantities of total anions. It appears that methanol is the basic extracting solvent of total anions in both systems.

Table 7. Comparison of Total Anion Concentrations

Solvent	Total Anions (μg/g) Soxhlet 24 h	Sonication 30 min	Sonication 2 h
CH	29.12	17.09	13.80
DCM	19.32	21.77	81.89
Ac	583.71	319.66	528.49
MeOH	992.67	878.54	1124.87
CH/MeOH		949.44	99.749
T:DCM:MeOH		443.69	500.17

Table 8 summarizes anion analysis of the four single solvents in the Soxhlet extraction technique. The major quantity of anion extracted for acetone is nitrate ion and for methanol is sulfate ion. Neither of these ions appreciably affect the microbial mutagenicity bioassay results. The results of further studies incorporating sonication and the comparative solvent systems are shown in Table 9 for nitrate ion and Table 10 for sulfate ion.

It should be noted that while the comparative solvent systems do not extract significantly more nitrate ion than does the single solvent acetone, they extract far less of the sulfate ion than does the single solvent methanol. Therefore, it appears that the two comparative solvent systems (CH/MeOH and T:DCM:MeOH) are not more effective than the Soxhlet-extracted single solvents (acetone and methanol).

Bioassay Results and Discussion

The *S. typhimurium* mutagenesis data for tester strain TA98 for each solvent system and extraction technique are shown in Table 11. DCM extraction, either by Soxhlet or sonication, resulted in an extractable material that was more mutagenic than that resulting from any of the other solvents. The least polar solvent, cyclohexane, was the least effective in extracting mutagens by either method. Acetone and methanol solvent extraction generally resulted in less-mutagenic samples than DCM extraction, except when acetone was used in sonication. Acetone removed considerably more extractable mass from air particles, including more inorganics. Therefore, when the mutagenic activity was

Table 8. Ambient Air Anion Analysis

	Anion Concentrations (µg/g)[a]			
Solvent	Fl	Cl	NO_3	SO_4
CH	0.02	2.6	7.0	19.5
DCM	0.02	2.2	2.8	14.3
Ac	2.41	83.7	477.5	20.1
MeOH	0.77	18.7	56.1	917.1

[a] Soxhlet extraction for 24 h.

Table 9. Ambient Air--Glass Fiber Filters Nitrate Analysis

	Nitrate (µg/g)		
Solvent	Soxhlet 24 h	Sonication 30 min	Sonication 2 h
CH	7.01	6.73	1.22
DCM	2.81	3.93	3.04
Ac	477.48	265.27	290.62
MeOH	56.11	42.92	498.57
CH/MeOH		505.48	468.43
T:DCM:MeOH		318.32	397.02

Table 10. Ambient Air--Glass Fiber Filters Sulfate Analysis

	Sulfate (μg/g)		
Solvent	Soxhlet 24 h	Sonication 30 min	Sonication 2 h
CH	19.48	7.61	8.82
DCM	14.33	15.48	71.74
Ac	20.06	12.61	4.26
MeOH	917.12	825.41	516.80
CH/MeOH		331.40	425.61
T:DCM:MeOH		51.54	6.88

Table 11. Mutagenic Activity of Different Solvent Extractable Material from Air Particulate in TA98 (without S-9 activation)

	Soxhlet Extraction		Sonication	
Solvent	Rev/μg	95% Confidence Limits	Rev/μg	95% Confidence Limits
CH	0.11	0.05 - 0.16	0.09	0.06 - 0.13
DCM	0.52	0.43 - 0.62	0.48	0.37 - 0.58
Ac	0.28	0.23 - 0.33	0.45	0.38 - 0.53
MeOH	0.26	0.23 - 0.30	0.35	0.28 - 0.41
CH/MeOH			0.38	0.30 - 0.45
T:DCM:MeOH			0.37	0.26 - 0.48
DMSO			0.07	0.03 - 0.10

calculated per milligram of particle, as shown in Table 12, acetone appeared to result in greater mutagenic activity. Consequently, the use of acetone may be advantageous for certain studies. Analytical problems associated with the use of acetone plus the presence of higher concentrations of inorganic salts have resulted in our selection of a solvent other than acetone for most studies.

Table 12. Ambient Air Particle Mutagenicity in TA98 (without S-9 activation)

Solvent	% Extractable[a]	Revertants/µg Extractable	Revertants/mg Particle
CH	4.0	0.11	4.4
DCM	6.4	0.52	33.3
AC	21.0	0.28	58.8
MeOH	8.9	0.26	23.1

[a] Soxhlet extraction.

While DMSO can be used directly both to suspend the particles and administer to the bioassay without evaporation or solvent exchange, it was the least effective method for detecting the mutagenic activity present. Thus, DCM is the preferable solvent, particularly for studies in which the amount of nonmutagenic mass should be minimized.

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INTEGRATION OF THE AMES BIOASSAY AND CHEMICAL ANALYSES IN AN EPIDEMIOLOGICAL CANCER INCIDENCE STUDY

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INTRODUCTION

The development of the Ames bioassay as an instrument for assessing public health problems involving mutagenicity and potential carcinogenicity has resembled the development of other quantitative techniques for assessing public health problems. The Ames test has developed from a qualitative assay of samples in simple matrices into a quantitative determination of complicated environmental mixtures, and its results are now being integrated with human epidemiological studies. Originally, the Ames test was used primarily to determine whether or not a compound was mutagenic (Ames, 1971). Soon quantitative methods were introduced (Ames et al., 1975), and a significant correlation between mutagenicity in the Ames test and carcinogenicity in animal bioassays emerged (McCann et al., 1975). Shortly thereafter, the test was applied to environmental mixtures in the analysis of air particulate material (Pitts et al., 1977; Talcott and Wei, 1977). Currently, the Ames test is used to detect mutagens in a variety of media and sample types. The rapidly expanding list of applications includes drinking water, cigarette smoke, auto exhaust, foods, drugs, and urine (Holstein et al., 1979). Although not a quantitative test in the sense of having well-established precision and accuracy, the Ames bioassay yields results that indicate relative mutagenicity. Thus, it is appropriate to consider its use in epidemiological cancer studies.

The application of the Ames mutagenicity test to epidemiological cancer studies is logical because mutagenicity is in some measure a composite index of total potential carcinogenicity (McCann et al., 1975). It can be argued that

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determining the concentrations of all carcinogens in the medium also should give, at least in theory, the information needed to determine the carcinogenic exposure to humans. However, chemical methods are not available to detect all possible carcinogens in a given medium. Furthermore, even an exhaustive compilation of carcinogens would neglect synergistic or antagonistic effects. Thus, application of both chemical and bioassay techniques more completely characterizes environmental samples (Bjϕrseth et al., 1980).

Contra Costa County, CA, was suspected of having high rates of respiratory-tract cancer, based on the findings of the national survey of cancer mortality by county, 1950 through 1969 (Mason and McKay, 1973). The northern section of Contra Costa County is heavily industrialized, with five major petroleum refineries and many petrochemical plants. These facts prompted the U.S. Environmental Protection Agency (EPA) to fund an epidemiological study of cancer incidence as related to airborne emissions in Contra Costa County. This study is part of a larger study funded jointly by the State of California and the Occupational Safety and Health Administration (OSHA). The larger study includes four other counties in the San Francisco Bay Area and is being carried out by the California Resource for Cancer Epidemiology (RCE). The present discussion is restricted to the Contra Costa County portion of the project. The major objectives of the study are 1) to identify environmental factors which may contribute significantly to cancer incidence in the county, 2) to determine whether various groups of workers are more likely to develop cancer than others, and 3) to evaluate whether air pollutants have affected the observed incidence of respiratory-tract cancer in the county.

An important component of the study is environmental monitoring, consisting of ambient air particulate matter sampling and subsequent chemical and biological analyses. A major goal is to determine whether or not mutagenic activity, as measured by the Ames assay, can be accounted for by the chemical characterization of the samples. Environmental monitoring will provide a means of correlating the geographic distribution of current cancer incidence with current ambient air pollutants and to develop baseline air pollution data for comparison with future measurements and use in future epidemiological cancer studies. Because of the latency of cancer onset, current incidence levels cannot be causally related to current air quality. Analysis of past and present emission data and air quality patterns could, however, reveal historical pollution trends useful in determining the association, if any, between air pollution and current cancer incidence.

This paper will discuss the design of the environmental monitoring program and preliminary data from the chemical and biological assays. The planned integration of this environmental data base with the epidemiological cancer study will be described.

PROCEDURE

The Sampling Program

Fifteen high-volume particulate samplers were placed at thirteen locations in Contra Costa County and two locations in adjacent counties (shown in Figure 1), in order to characterize air quality variations over the entire county. Five of the locations are permanent stations of the Bay Area Air Quality Management District (BAAQMD). Although these five stations monitor for several pollutant gases as well as for particulate matter, the present paper will discuss only particulate matter results.

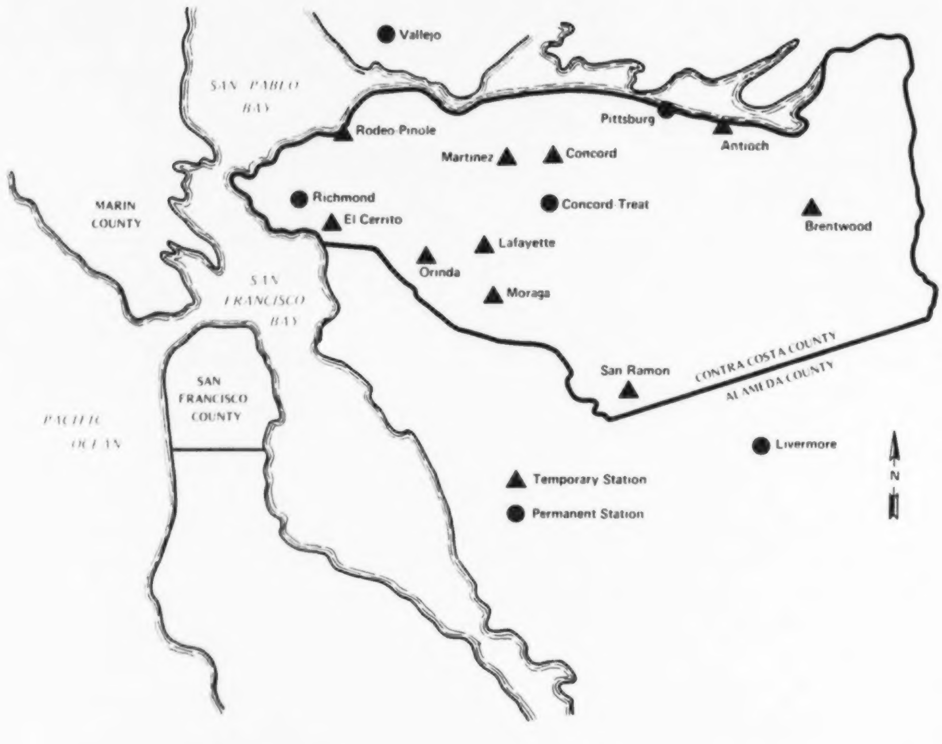
Vallejo
SAN PABLO BAY
Rodeo Pinole
Pittsburg
Antioch
Martinez
Concord
MARIN COUNTY
Richmond
El Cerrito
Concord Treat
Brentwood
Lafayette
Orinda
Moraga
SAN FRANCISCO BAY
SAN FRANCISCO COUNTY
PACIFIC OCEAN
San Ramon
CONTRA COSTA COUNTY
ALAMEDA COUNTY
Livermore
▲ Temporary Station
● Permanent Station
N

Figure 1. Locations of sampling stations in Contra Costa County, CA.

Air particulate material was collected on 8- x 10-in. (20- x 25-cm) glass-fiber filters (EPA Grade Whatman) in standard high-volume samplers, which drew approximately 1,200 m^3 of air through a filter during each 24-h run. Filters were collected every sixth day at each of the 15 sampling stations, from November 3, 1978, through October 31, 1979. In this period, nearly 900 air-filter samples were collected and analyzed.

Particulate matter was analyzed for benzene-soluble organics (BSO), lead (Pb), total suspended particulate matter (TSP), nitrates (NO_3^-), sulfates ($SO_4^=$), specific polycyclic aromatic hydrocarbons (PAH), and mutagenic activity, using the Ames test. The BAAQMD collected the air samples and analyzed them for TSP, NO_3^-, and $SO_4^=$, and the Air and Industrial Hygiene Laboratory (AIHL) carried out the other analyses.

Logistics of Sample Analysis and Data Management

The plan for distributing filter samples for analysis and reporting results is shown in Figure 2. After weekly sample collection, the filters were weighed to determine the amounts of total suspended particulate material and were delivered to AIHL. There, the filters were logged in and cut, and the pieces were distributed for further analysis.

The crux of the air-monitoring program is the analysis of composite air samples from each of the 15 stations for PAH content and mutagenic activity. For each station, composite samples were prepared by combining samples collected over each of the following four-month periods: November, 1978, through February, 1979 (winter); March through June, 1979 (spring); and July through October, 1979 (summer). These three periods correspond to the three meteorological seasons of the San Francisco Bay Air Basin. Filter disks for PAH analysis and mutagenicity testing collected between November 1, 1978, and May 1, 1979, were stored in the dark at room temperature. Filters collected between May 1 and October 31, 1979, were stored in the dark at -20°C. Disks (47-mm diam.) were cut from each filter and individually extracted ultrasonically with organic solvents. For each location, aliquots taken during a given four-month period were combined to provide a composite sample for PAH and mutagenic analysis.

Analysis Methods for TSP, BSO, Pb, NO_3^-, and $SO_4^=$

Standard methods were used to analyze for the following five pollutants: TSP was determined gravimetrically (BAAQMD, 1977); NO_3^- colorimetrically (BAAQMD Method N-7, 1976); $SO_4^=$ turbidimetrically (BAAQMD Method S-42, 1976); BSO by Soxhlet

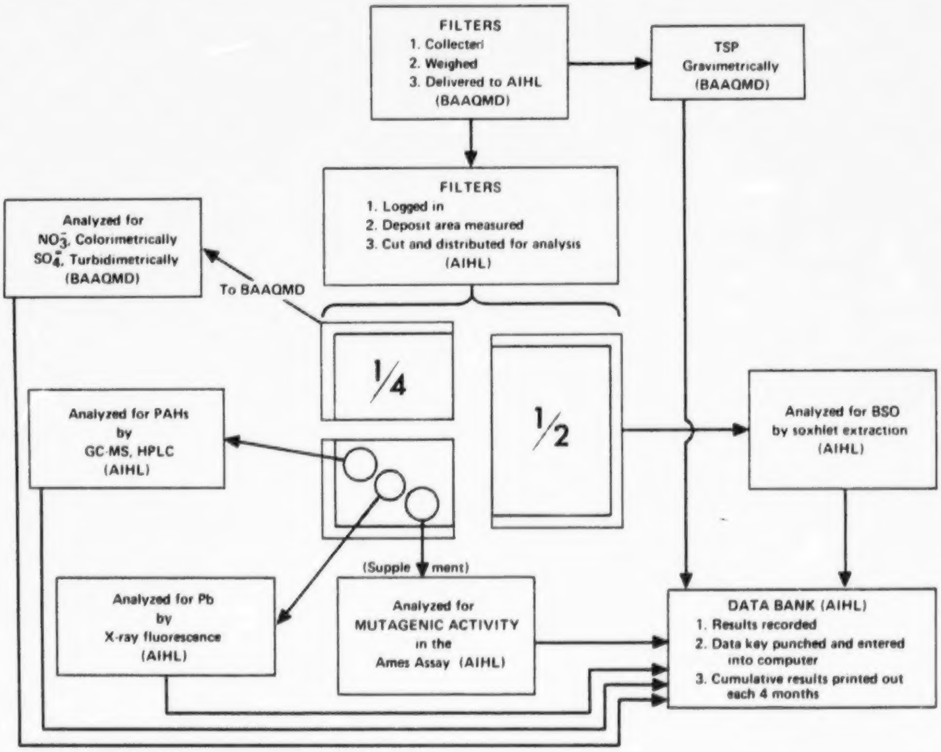
FILTERS
1. Collected
2. Weighed
3. Delivered to AIHL
(BAAQMD)
TSP
Gravimetrically
(BAAQMD)
FILTERS
1. Logged in
2. Deposit area measured
3. Cut and distributed for analysis
(AIHL)
Analyzed for
NO_3^-, Colorimetrically
$SO_4^=$, Turbidimetrically
(BAAQMD)
To BAAQMD
1/4
1/2
Analyzed for PAHs
by
GC-MS, HPLC
(AIHL)
Analyzed for BSO
by soxhlet extraction
(AIHL)
(Supplement)
Analyzed for Pb
by
X-ray fluorescence
(AIHL)
Analyzed for
MUTAGENIC ACTIVITY
in the
Ames Assay (AIHL)
DATA BANK (AIHL)
1. Results recorded
2. Data key punched and entered into computer
3. Cumulative results printed out each 4 months

Figure 2. Logistical plan for analysis of high-volume air filters collected in Contra Costa County, CA, November, 1978, through October, 1979.

extraction (AIHL, 1975); and Pb by wavelength dispersive X-ray fluorescence (Moore, 1976).

Analysis Methods for PAH

Analysis methods for PAH were modified from Bjørseth et al. (1980). Individual (47-mm) filter disks were placed in screw-cap test tubes and extracted twice at 40 to 45°C for 20 minutes, first with 8 ml and then with 6 ml of cyclohexane (MCB, OmniSolv) in an ultrasonic bath (Bransonic Models 220 or 32). Each cyclohexane extract was filtered through a 0.5-μm Fluoropore filter (Millipore). Extracts comprising each composite were combined in a round-bottom evaporating flask and concentrated to 6 ml in a rotary evaporator at 45 to 50°C. To separate PAH from interfering material, the 6 ml of concentrated cyclohexane extract was combined with 2 ml of toluene (MCB, OmniSolv) and extracted ultrasonically with 12 ml of a 10:1 mixture of N,N-dimethylformamide (DMF) (MCB Manufacturing Chemists, Inc., OmniSolv) and water in a screw-cap test tube for 15 min. The bottom layer, containing the PAH, was transferred by pipette to a 60-ml separatory funnel. The remaining cyclohexane phase was re-extracted ultrasonically twice more with 6 ml of the DMF-H_2O mixture, and the phases containing the PAH were combined in the separatory funnel. Following the addition of 24 ml of distilled water to the separatory funnel, the 2 ml of toluene, containing the PAH, separated. The toluene phase was transferred to a centrifuge tube and evaporated to dryness in a heating block at 45°C under a stream of nitrogen. The residue was then dissolved in 200 to 400 µl of acetonitrile for analysis by high-pressure liquid chromatography (HPLC).

A Varian Model 5000 high-pressure liquid chromatograph and Microbondapak C18 (Waters Associates) column were used to separate PAH. Column effluents were monitored using ultraviolet (UV) absorption (at 254 nm) and fluorescence (excitation, 263 nm; emission, 407 nm). Fluorescence measurements were used to resolve and quantitate three carcinogenic PAH: benz(a)anthracene, benzo(a)pyrene, and chrysene. Fluorescence measurements were made with a Perkin-Elmer Model MPF-44A spectrofluorometer. HPLC was performed in a linear gradient from 70% acetonitrile in water to 100% acetonitrile, in 50 min. The flow rate was 0.8 ml/min, the temperature was 30°C, and the chart speed was 1 cm/min. The injection was made with a sample loop operated by a rotary valve, using a 10-µl injection volume.

The efficiency of extraction of PAH from high-volume filters has been studied. Fluorescence measurements show that more than 95% of PAH can be recovered from a spiked filter.

Peaks observed in the HPLC chromatograms were identified by three methods. First, peaks were tentatively identified by comparing their retention times to those of standards. Second, the peak height ratios of samples and standards were compared at the wavelengths used to measure absorbance and fluorescence. Identifications of the three PAH were confirmed by stopping the flow during HPLC analysis and scanning the fluorescence spectra using the optimum excitation wavelength for each compound. Peak heights were used for quantitation of PAH.

The selectivity of the fluorescence detection method is illustrated in Figures 3 and 4. Using fluorescence excitation and emission wavelengths of 263 nm and 407 nm respectively, most of the UV-absorbing PAH peaks were suppressed, but benzo(a)pyrene, benz(a)anthracene, and chrysene were enhanced. Such specificity is critical, because many poorly resolved peaks, including those containing the 10 PAH standards, are visible in the chromatograms of air samples using UV detection (Figure 4). The major peak eluting just before phenanthrene was due to an organic contaminant in the cyclohexane used in early experiments. This artifact disappeared when the brand of cyclohexane specified above was substituted.

The Ames Test for Mutagenic Activity

Methods for extracting air particulate material from high-volume glass-fiber filters and making composite samples were adapted from Pitts et al. (1979). The solvent, a 1:1:1 mixture of methanol, dichloromethane, and toluene (MCB, OmniSolv), was prepared fresh daily and saturated with nitrogen. Extractions were carried out in an ultrasonic bath under low light. Each individual 47-mm filter disk cut from a filter was placed in a 16- x 125-mm screw-cap tube with a Teflon liner, and 4 ml of solvent was added. Tubes were sonicated for 20 min at maximum power in the ultrasonic bath containing water at 45°C. Extracts were filtered through 0.5- m Fluoropore filters (Millipore); filter disks were re-extracted using 3 ml solvent, and the filtrates were combined. The volume of each extract was adjusted to exactly 10 ml with the solvent, and the extracts were stored at -20°C.

Composite samples for mutagenic testing were prepared by combining aliquots of stored extracts in a vacuum flask, saturating the extracts with nitrogen, and reducing the volume of solvent in a rotary evaporator, under reduced pressure and at a temperature of 45°C. The composite samples were then transferred to preweighed tubes, which were placed in a heat block at 45°C; the remaining extraction solvent was removed under a stream of nitrogen. After weighing, residues were redissolved in dimethylsulfoxide for mutagenic analysis.

The method for detecting mutagens with the *Salmonella*/mammalian microsome test was as described by *Ames et al.* (1975), with the following changes: rat liver homogenate (S-9) was prepared from rats fed commercial rodent foods; rats were anesthesized with carbon dioxide before surgery; and plates were incubated for 72 instead of 48 h. S-9 protein concentrations were determined by the method of Lowry et al. (1951).

Negative solvent (dimethylsulfoxide) and S-9 sterility controls and positive controls for each strain used were run with each experiment. The control mutagens for the five tester strains were sodium azide in TA1535; 9-aminoacridine in TA1537; 2-aminofluorene in TA1538 and TA98; and methyl methanesulfonate in TA100. The Ames assay was applied according to a two-part protocol (Pitts et al., 1979). The first step involved screening the sample in the five standard Ames tester strains both with and without metabolic activation. These data gave a qualitative estimate of

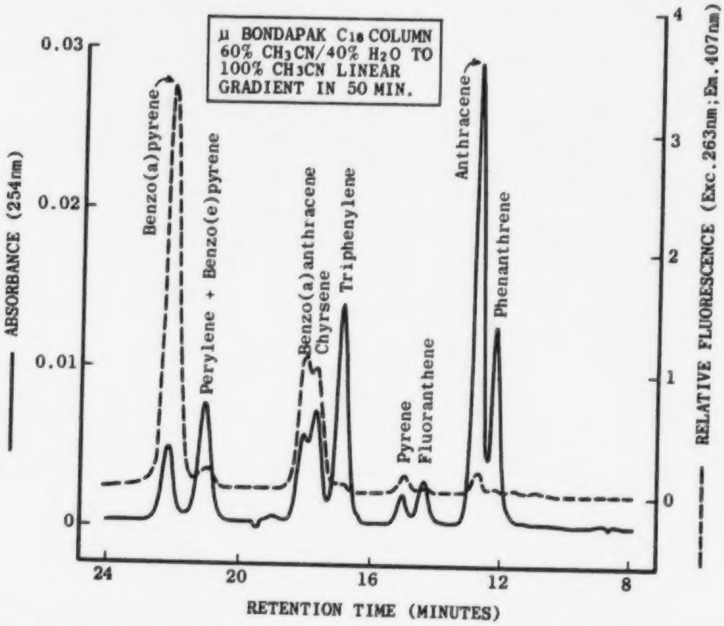

Figure 3. High-pressure liquid chromatogram of a 10-PAH standard detected by fluorescence and UV absorbance.

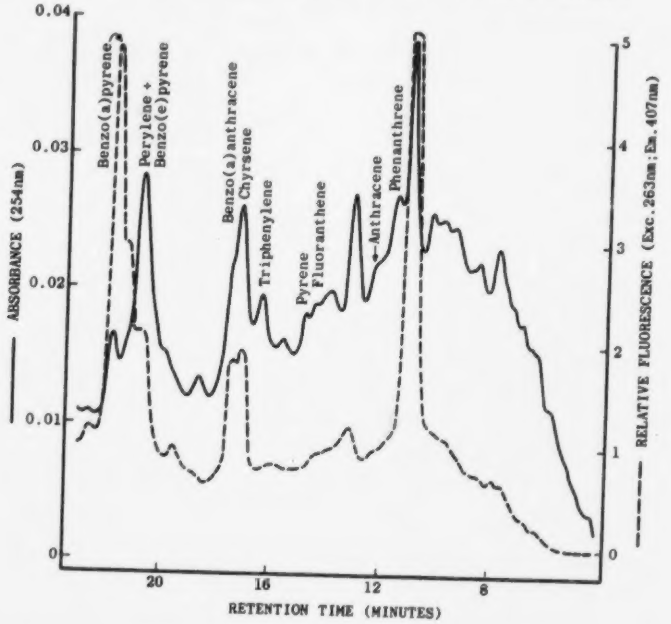

Figure 4. High-pressure liquid chromatogram of the PAH fraction from air particulate material collected in Antioch, CA, November, 1978, through February, 1979.

the mutagenic activity and indicated the most sensitive strain and the optimum conditions of metabolic activation for subsequent quantitative analysis. For initial screening, each composite sample was assayed at one dose in the range 20 to 1000 µg/plate. Determinations were made with and without added rat liver S-9 at both low (~ 0.6 mg/plate) and high (~ 3 mg/plate) protein concentrations.

All composite samples exhibited mutagenic activity in the initial screening and were reanalyzed in the strain showing the greatest response. The dose range and conditions of metabolic activation also were chosen to maximize activity. Duplicate determinations were made at each of several doses and the result expressed as revertants per cubic meter.

An interlaboratory comparison of the mutagenicity of ambient particulates was carried out between AIHL and the Statewide Air Pollution Research Center, University of California, Riverside. High-volume filter samples collected in southern and northern California were split and analyzed for mutagenic activity in the Ames test. The determinations made by the two laboratories agreed within a factor of two.

RESULTS

As analyses are still in progress, only partial results are presented.

Table 1 gives the median and maximum values for TSP, BSO, and Pb for winter and spring. The median and maximum levels of the three pollutants were the highest in winter, when meterological inversions frequently occur. The extreme values for individual 24-h runs differed by a factor of 50. For example, the highest 24-h level of BSO was 41.3 mg/m^3, in Concord in early December, 1978, and the lowest was less than 0.8 mg/m^3 (the detection limit) at several sites in January and February, 1979. The highest Pb concentration was found in Antioch, also in early December, while the highest TSP concentration was in Brentwood in November.

To describe variations in levels of community air pollution throughout the county, maps showing the geographical distribution of the seven measured pollutants are being constructed using a computer program called SYMAP (developed by the Laboratory for Computer Graphics, Harvard University, Cambridge, MA). In this program, sampling station coordinates and associated pollutant levels are used to construct a matrix containing the pollution levels at each station. Contours are constructed by interpolation. These distributions will be used to estimate community exposure levels and will be compared with the patterns of cancer discovered

Table 1. Analysis of Air Particulate Material Collected in Contra Costa County, CA

	24-Hour Median Value		24-Hour Maximum Value	
Pollutant	Nov. 1978-Feb. 1979	March-June 1979	Nov. 1978-Feb. 1979	March-June 1979
Total suspended particulate material	60	42	229	126
Benzene-soluble organics	4.8	1.4	41.3	32.1
Lead	0.7	0.2	2.5	0.6

in epidemiological studies. The distributions may also provide clues to pollution sources.

Thus far, computer-drawn contour maps of TSP mass, BSO, and Pb levels for winter and spring have been prepared; these are shown in Figure 5. They were constructed using average values obtained at each sampling station during the first two seasons for which composite samples were analyzed. Panels A, B, and C show the distributions of these pollutants during winter; panels D, E and F show the distributions in the spring. Concentrations of the pollutants were generally higher in winter than in spring, and seasonal variations were most pronounced for Pb and BSO levels, which changed by more than a factor of three (see Table 1). The geographical distributions of Pb and BSO were similar. For both pollutants, the highest levels were found in winter in a north-south band located in central Contra Costa County. This region corresponds roughly to the Diablo Valley, a natural pollution sink through which runs a major freeway.

At present, three PAH have been quantitated: benz(a)anthracene benzo(a)pyrene, and chrysene. Concentrations from the Antioch and Brentwood winter composite samples are listed in Table 2. Antioch is an urban-industrial site; Brentwood is a rural location. The range of concentrations of benz(a)pyrene found in these samples (0.1 to 0.8 ng/m) was comparable to, although somewhat lower than, that found in particulate samples collected in San Francisco 20 years ago (Sawicki et al., 1960). The present values were also

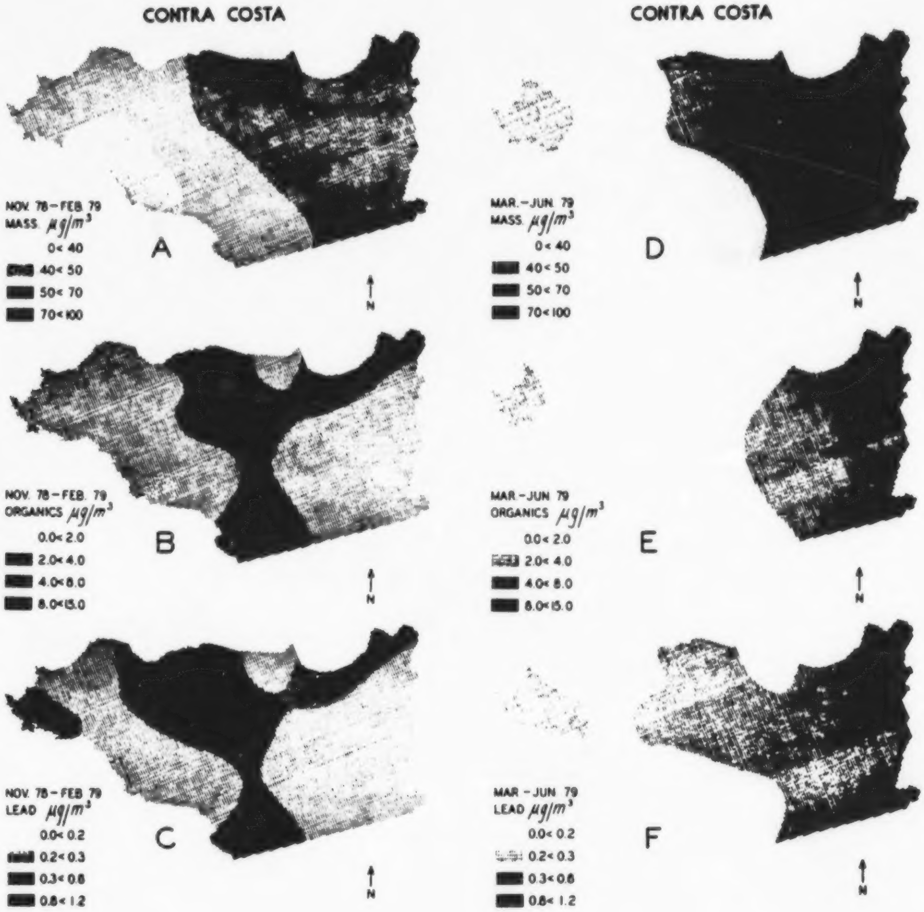
CONTRA COSTA
CONTRA COSTA
NOV. 78 – FEB. 79
MASS μg/m³
0< 40
40< 50
50< 70
70<100
A
N
MAR. – JUN. 79
MASS μg/m³
0< 40
40< 50
50< 70
70<100
D
N
NOV. 78 – FEB. 79
ORGANICS μg/m³
0.0< 2.0
2.0< 4.0
4.0<8.0
8.0<15.0
B
N
MAR. – JUN. 79
ORGANICS μg/m³
0.0< 2.0
2.0< 4.0
4.0< 8.0
8.0<15.0
E
N
NOV. 78 – FEB. 79
LEAD μg/m³
0.0< 0.2
0.2< 0.3
0.3< 0.8
0.8< 1.2
C
N
MAR. – JUN. 79
LEAD μg/m³
0.0< 0.2
0.2< 0.3
0.3< 0.8
0.8< 1.2
F
N

Figure 5. Computer-drawn contour maps of the geographical distribution of levels of total mass, benzene-soluble organics, and lead in air particulate material collected in Contra Costa County, CA. A, B, and C show distributions for November, 1978 through February, 1979, and D, E, and F show March through June, 1979, distributions.

comparable to those measured more recently in New York City (Daisey et al., 1979) and Germany (Gusten and Heinrich, 1978).

Extracts from samples collected at the 15 stations have been analyzed qualitatively for mutagenicity, and all showed activity in

Table 2. Polycyclic Aromatic Hydrocarbon Content and Mutagenic Activity in Contra Costa County Air Particulate Material Collected November, 1978, through February, 1979

Type of Measurement	Sampling Location	
	Antioch	Brentwood
PAH (ng/m^3)		
Benz(a)anthrene	0.81	0.10
Benzo(a)pyrene	0.81	0.10
Chrysene	0.90	0.16
Sum of three PAH	2.52	0.36
Mutagenic activity (revertants/m^3)		
Without S-9	6.3	3.9
With S-9	25.4	5.9

at least one strain. The most activity was seen in strains TA98 and TA1538. Adding the S-9 fraction generally enhanced activity; some samples were most active at the high S-9 protein concentration (3 mg/plate), while the majority were most active at the lower concentration (0.6 mg/plate). Dose-response curves for the Antioch and Brentwood composites (with activities given as revertants/plate) are shown in Figures 6 and 7. Over the dose range used (up to 20 m^3 of air), the mutagenic responses appear linear. The dose-response curves obtained with the Antioch winter composite (Figure 6) were somewhat atypical, in that most samples did not show a fourfold increase in activity in the presence of S-9. The response to S-9 in samples analyzed to date was more typically that shown by the Brentwood sample (Figure 7), although stations in more heavily polluted areas generally showed greater S-9 enhancement.

Seasonal variations in mutagenic activity were also observed. Activities (expressed as revertants per cubic meter) were generally higher for samples collected in winter than for samples collected in spring or summer. Table 2 summarizes results from the Antioch and Brentwood Stations. Samples from Antioch, a more urban setting than Brentwood, showed both higher concentrations of PAH and

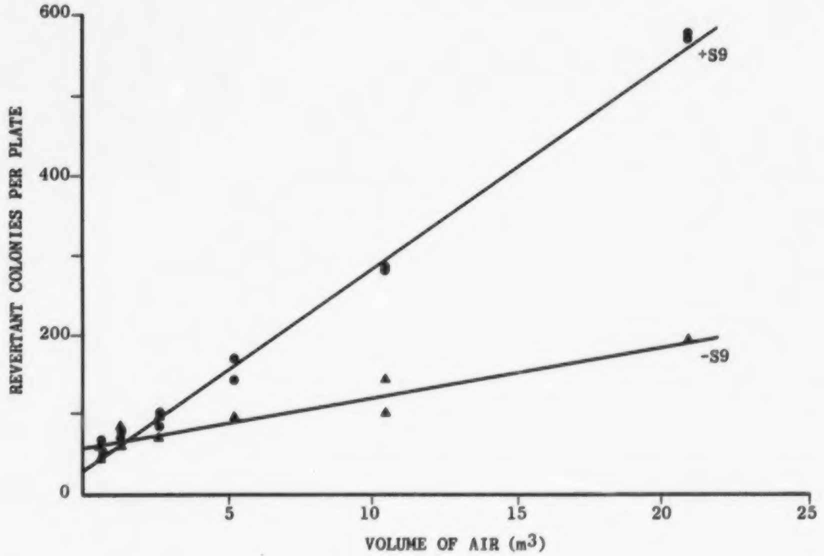

Figure 6. Ames test dose-response curves for a composite sample from Antioch, CA, November, 1978, through February, 1979, assayed in strain TA98, with and without 3 mg S-9 protein/plate.

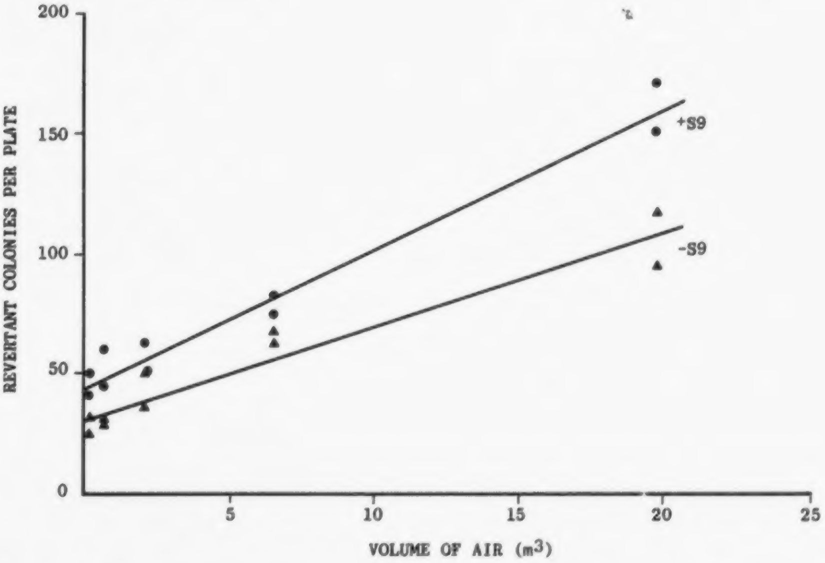

Figure 7. Ames test dose-response curves for a composite sample from Brentwood, CA, November, 1978, through February, 1979, assayed in strain TA98, with and without 0.6 mg S-9 protein/plate.

increased mutagenic activity. The activities and the urban-rural differences found in this study were similar to those found previously in California (Flessel, 1977; Pitts et al., 1977, 1979).

One can also compare chemical composition and biological activity in a given sample. For example, the Antioch sample analyzed above had a benzo(a)pyrene concentration of 0.81 ng/m^3. As the molecular weight of benzo(a)pyrene is 252, this corresponds to approximately 0.003 nmol/m^3. The specific mutagenic activity of benzo(a)pyrene is approximately 121 revertants/nmol (Ames et al., 1975). Therefore, the amount of benzo(a)pyrene measured accounts for about 0.36 revertants/m^3, or less than 2% of the observed activity. Clearly, chemicals other than benzo(a)pyrene account for most of the mutagenicity in this and other air samples (Bjørseth et al., 1980).

DISCUSSION

The cancer epidemiology project plan consists of a series of studies, both prospective and retrospective, to investigate environmental factors relevant to cancer in Contra Costa County. The study will include a census tract analysis of cancer incidence at specific sites and is designed to ultimately attempt to distinguish between the contributions of occupational and community exposures to the incidence of cancer. From the mutagencity and PAH data, community exposures in Contra Costa County will be estimated. These exposures will be examined for correlations with present and future cancer rates in various geographical areas. Although this is fundamentally a prospective study, attempts will be made to interpret current cancer incidence rates in terms of current exposures and historical pollution trends. Definitive studies will require following current Contra Costa residents through the next several decades and monitoring a larger number and variety of carcinogens and mutagens in community air. It might be worthwhile to expand monitoring activities to include data from community water, soil, food, and workplace samples.

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Levaggi, W. Siu, D. England, and N. Balberan (BAAQMD); and D. Austin, W. Mandel, and S. Lum (RCE, California Department of Health Services).

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MUTAGENICITY OF AIRBORNE PARTICULATE MATTER IN RELATION TO TRAFFIC AND METEOROLOGICAL CONDITIONS

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Central Institute for Industrial Research
Oslo, Norway

INTRODUCTION

It is now well established that environmental factors are major causes of cancer in man (Higginson and Muir, 1973). Epidemiological studies have shown that the incidence of lung cancer is higher in urban than in rural areas (Henderson et al., 1975). Urban air contains large amounts of particulate pollutants, which are believed to contribute to this higher lung-cancer incidence (Menck et al., 1974). Furthermore, the carcinogenic potential of organic extracts from airborne particles has been demonstrated in animal experiments (Hueper et al., 1962). These observations have made it increasingly important to identify carcinogenic compounds in ambient air. Since animal studies are expensive and time-consuming, short-term tests for mutagenicity in microbial systems are currently used to identify possible carcinogens.

The presence of mutagenic compounds in airborne particulate matter from urban and industrial areas has been documented by us and others using the Ames Salmonella test system (Dehnen et al., 1977; Löfroth, 1978; Møller and Alfheim, 1980; Pitts et al., 1977; Talcott and Wei, 1977; Teranishi et al., 1978; Tokiwa et al., 1977). Mutagenic activity of airborne particulates from a rural site was investigated in only one of these studies; all of the rural samples were reported to be inactive (Pitts, 1977). Part of the observed mutagenic activity in samples from urban air is probably due to polycyclic aromatic hydrocarbons (PAH). Several PAH compounds cause cancer in animals and are also suspected of causing cancer in man. However, these compounds require metabolic activation before they can act as mutagens in the Salmonella test

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system (Ames et al., 1973). In the studies cited, all urban samples were mutagenic both with and without metabolic activation, indicating the presence of mutagens other than PAH.

Mutagens in ambient air originate from various combustion sources, including residential heating and motor vehicle exhausts (Møller and Alfheim, 1980; Löfroth, 1979; Wang, 1978), and may be transported over long distances (Alfheim and Møller, 1979). Many parameters will influence the mutagenicity of airborne particulate matter from a given source, including the distance from the source, meteorological conditions, the presence of other pollutants, and the location of the source. In this work, we compared the mutagenicity of urban air particulate matter at street level to that at roof level, to determine the contribution of traffic to the mutagenicity of urban air. We also compared the mutagenicity of urban and rural airborne particulate matter, and we related mutagenic activity to meteorological conditions and to the composition of the particulate matter.

MATERIALS AND METHODS

Sampling

The urban sampling sites were all located in the center of Oslo, Norway. Two sampling sites were in a narrow street with heavy traffic (averaging 2000 cars/h during the day and 500 cars/h at night). Site A, street level, was 2 m above the ground, and site A, roof level, was 25 m above the ground. Sampling site B was in a park with much lower traffic frequency than at site A. Roof samples were taken at two other locations in Oslo: sampling site C was at a junction with heavy traffic, and site D was located in a more industrial area with less traffic. Sites A and C were considered to be more polluted than sites B and D, based on SO_2 and soot measurements. Rural samples were collected in southern Norway near the coast at Birkenes (site E) and in central Norway in a mountainous area at Hummelfjell (site F).

Airborne particulate matter was collected during the winter and spring of 1978 and 1979. Samples were collected on glass fiber filters (Gelman type A-E) with high-volume samplers. At sites A and B, the air was also passed through plugs of polyurethane (PUR) to adsorb the more volatile compounds (especially volatile PAH) (Alfheim et al., 1977). Separate day and night samples were collected, each during two 12-h periods (~400 m^3 air). At sites C and D, approximately 700 m^3 air passed through each filter during a 24-h period. About 2000 m^3 were sampled at the rural sites. For particle fractionation, a Sierra High Volume Cascade Impactor Sampler with split filters (Gelman type C-230) was connected to the sampler.

Extractions and Mutagenicity Testing

The organic compounds were extracted from the filters with 50 ml of acetone or 50 ml of cyclohexane, for nonpolar compounds, in a Soxhlet apparatus for 16 h. For mutagenicity testing, the samples were either concentrated by evaporation and tested directly or were evaporated to near dryness and dissolved in dimethylsulfoxide. Samples from locations A and B were extracted with acetone, and samples from location C, D, E, and F were extracted with cyclohexane. Acetone or cyclohexane extracts from unused filters were tested as controls; positive controls were 2-aminoanthracene and benzo(a)pyrene (B[a]P).

Salmonella typhimurium strains TA98 and TA100 were kindly supplied by Dr. B.N. Ames, University of California at Berkeley. Liver homogenate fractions were prepared from male Wistar rats injected with Aroclor 1254, 500 mg/kg i.p., five days prior to preparation. The assay was carried out as described by Ames et al. (1975).

Analysis of PAH

PAH were analyzed by the procedure of Grimmer and Bohnke (1972) as modified by Bjϕrseth (1977). Internal standards were added to the cyclohexane before extraction of the filters. The extracts were shaken once with 50 ml and once with 25 ml dimethylformamide (DMF):water (9:1). The DMF:water phases were separated, water was added to give a DMF:water ratio 1:1, and the samples were re-extracted with cyclohexane. The final cyclohexane extracts were concentrated, first with a modified Vigreux column under nitrogen and reduced pressure and then with a stream of highly purified nitrogen at 30°C. The analysis was performed on a Carlo-Erba Fractovap 2101 AC gas chromatograph with a glass capillary column and flame ionization detector. The cyclohexane extract, 2 μl, was injected splitless (Grob and Grob, 1969).

Chemical Fractionation

Acetone extracts from three samples collected at site A, street level, were pooled, evaporated to near dryness, transferred to diethylether, and fractionated into acidic, basic, and neutral fractions. The acidic and basic fractions contained compounds that were extractable from ether by aqueous sulfuric acid and sodium hydroxide, respectively, and re-extractable into ether after neutralization. The neutral fraction was further separated on a silica column by elution with cyclohexane, benzene, and ether. The cyclohexane fraction was subdivided into three fractions, on a column of silica gel with a layer of aluminum oxide on top, by elution with pentane containing increasing amounts of ether.

One sample from each of these seven fractions was evaporated to dryness and tested for mutagenic activity. The most mutagenic fractions were analyzed by glass capillary gas chromatography, as described above, or by combined gas chromatography/mass spectrometry (GC/MS) (Bjørseth et al., 1977).

RESULTS

Mutagenicity Testing

Preliminary results revealed very little or no mutagenic activity in Salmonella strain TA100; therefore, strain TA98 was used for further studies, including all those reported here. This strain had 35 to 45 spontaneous revertants per plate. Twice the number of spontaneous mutants was considered a significant mutagenic response.

Mutagenic substances either act directly or require metabolic conversion to mutagenic products. Unsubstituted PAH compounds require activation with mammalian enzymes to be mutagenic in the Ames test (Ames et al., 1973). The relative contributions of these two groups of substances were estimated by testing the extracts in both the presence and absence of liver microsomal preparations.

All extracts of airborne particulate matter collected on glass fiber filters were mutagenic both with and without metabolic activation. The dose response, expressed as number of mutants per plate, was linear both with and without S-9. The standard amount of S-9, 50 μl per plate, produced maximum activity for most samples, but more S-9 was needed for a few extracts. Extracts from the polyurethane filters showed no or very weak mutagenic responses. Some samples taken at street level were fractionated according to particle size. The results showed that only extracts from particles less than 2.7 μm were mutagenic in both the presence and absence of S-9.

Figure 1 shows the mutagenicity results for samples from site A, street and roof levels, and from site B, expressed as revertants per cubic meter of air. Daytime samples taken at street level were about twice as mutagenic with microsomal activation as without. (Mean values in February were 69 and 38 revertants/m^3 with and without S-9, respectively.) The activity of daytime samples collected at site A, roof level, was approximately the same with and without S-9; the same was true for site B daytime samples. These samples were only 5 to 25% as mutagenic as the site A street-level samples. For site A street-level samples, the

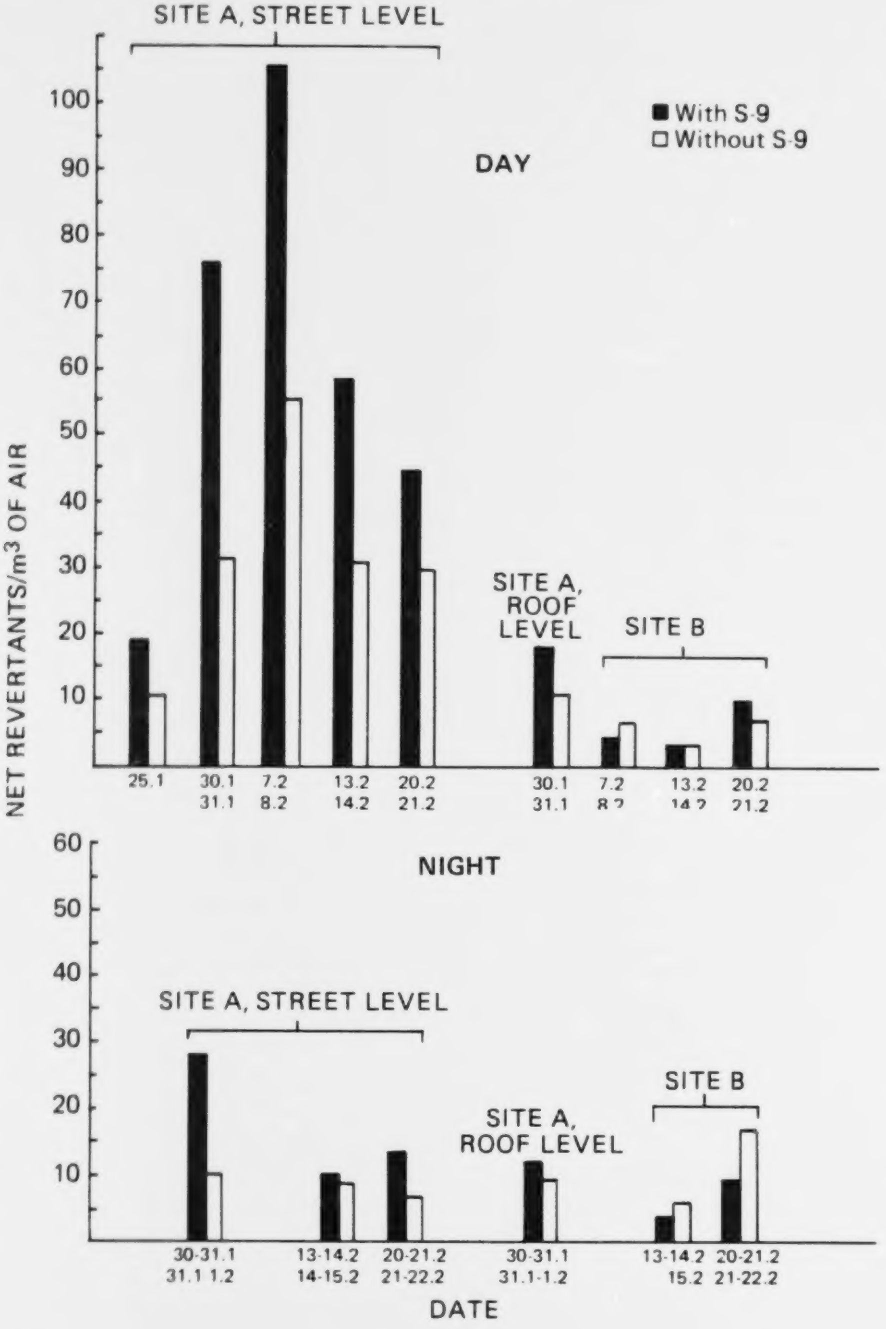

Figure 1. Mutagenicity of samples from site A (street-level and roof-level samples) and site B, expressed as revertants per cubic meter of air.

mutagenicity at night was only 20 to 25% of that during the day. The activity of extracts from site A, roof level, and from site B was relatively constant from one day to the following night.

The daily variation in the mutagenicity of nonpolar extracts sampled at sites C and D is shown in Figures 2 and 3. The mutagenicity of the samples from both locations was of the same magnitude (expressed as revertants per cubic meter of air in Figure 2). However, when expressed as revertants per milligram of particulate matter, the mutagenicity was more than twice as high at site D than at site C (Figure 3). The site C extracts were more mutagenic in the winter (February) than in the spring (April), both with and without metabolic activation.

The mutagenicity of urban air samples was compared to that of samples collected at site E, on the southern coast of Norway, far from any source of pollution. Samples from this location were mutagenic in both the absence and presence of metabolic activation (S-9) (see Figure 4). The highest mutagenic activity at site E was obtained for samples collected during the winter. Furthermore, the activity was significantly lower in samples representing air masses coming from the north than in samples representing air masses coming from the south. The mutagenic activity of these samples (expressed as revertants per cubic meter of air) was 5 to 10% of that for urban samples collected during the same period (sites C and D). The samples from site F, a rural inland site in the mountains, far from any pollution source, did not show any mutagenic activity. These nine samples were all made during the winter.

Chemical Fractionation of Street Samples

To characterize the mutagenic compounds in urban air, a few Oslo samples were fractionated, and the fractions were tested for mutagenicity. The results are given in Table 1. Only 48% of the mutagenicity assayed in the presence of S-9 was recovered after fractionation. Without S-9, the recovery was 30%. The mutagenic activity of the combined fractions was approximately equal to the sum of the activity of the individual fractions, indicating that there were no synergistic effects between substances found in different fractions.

The mutagenicity of the fractionated street-level samples was found mainly in the neutral aromatic fraction (N-2). Some activity was also associated with the acidic and the neutral fractions (N-4). The GC analysis showed that the N-2 fraction contained all the common PAH except for the most volatile compounds. In addition, this fraction contained unidentified polynuclear aromatic

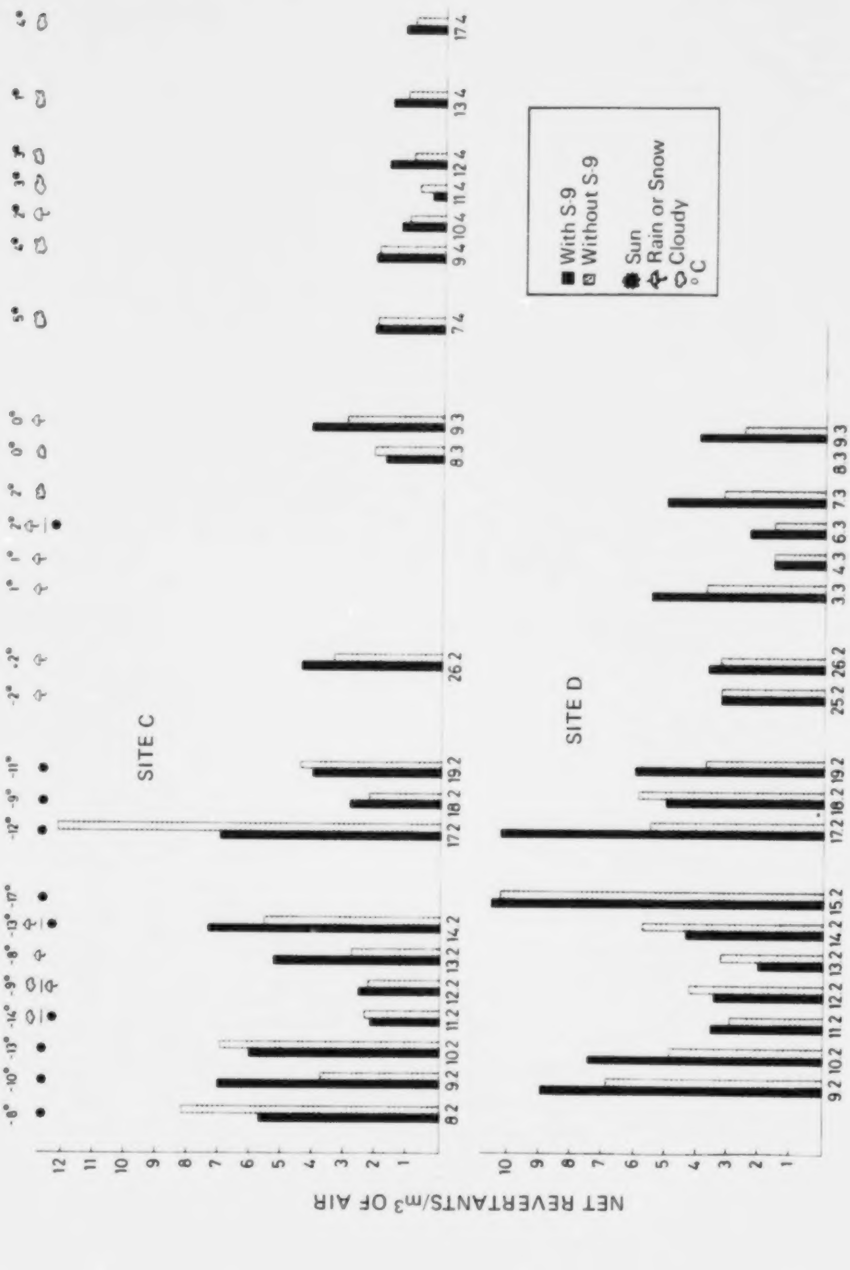

Figure 2. Mutagenicity of roof-level samples from sites C and D, expressed as revertants per cubic meter of air, and weather conditions on dates of sampling.

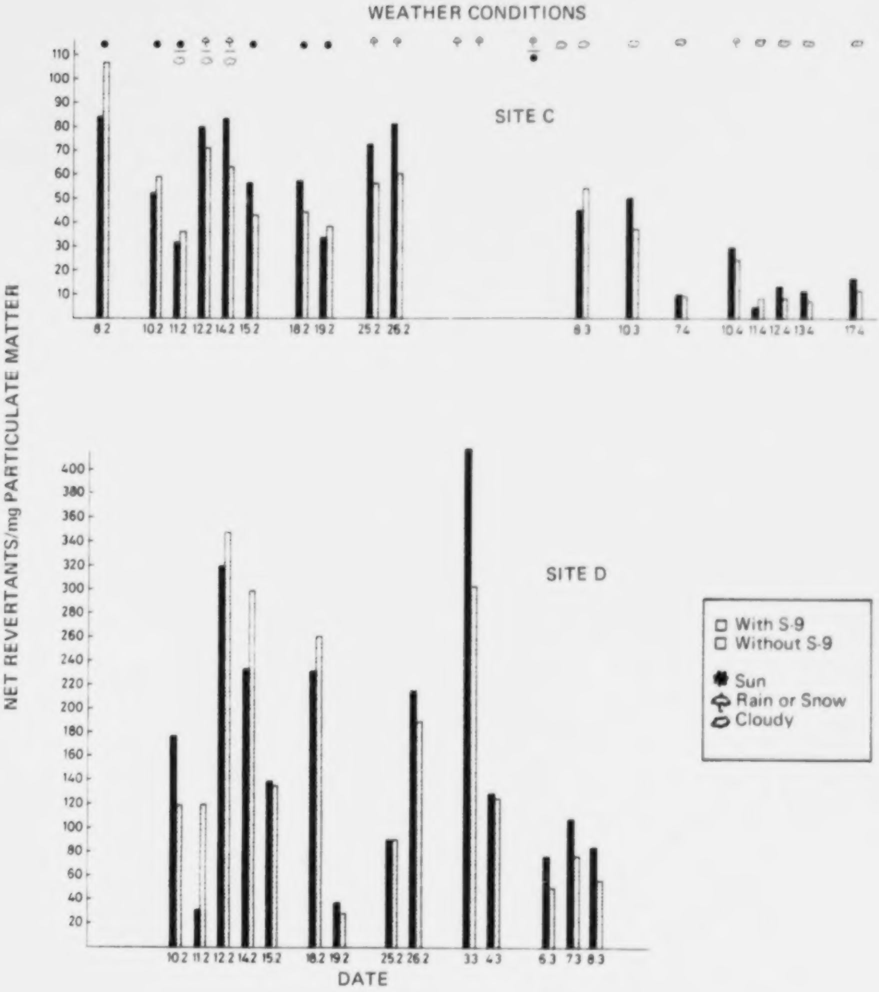

Figure 3. Mutagenicity of roof-level samples from sites C and D, expressed as revertants per milligram particulate matter, and weather conditions on dates of sampling.

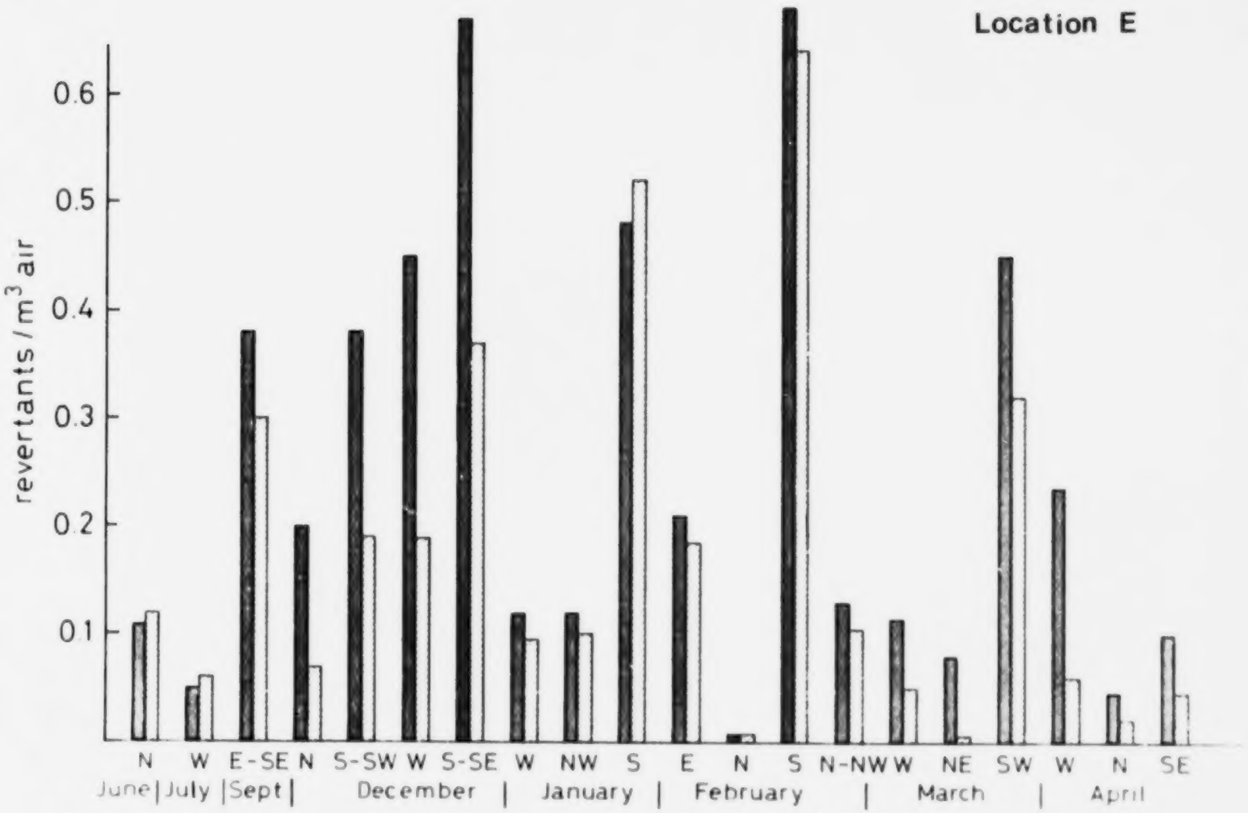

Figure 4. Mutagenicity of samples from site E, expressed as revertants per cubic meter of air. The main directions of the air masses during sampling are indicated as N = north, W = west, E = east, and S = south.

Table 1. Distribution of Mutagenicity Among Fractions of Extracts of Airborne Particulate Matter[a]

Fraction	Net Revertants/m^3 of Air	
	With S-9	Without S-9
Unfractionated	59	35
Acidic	5	2
Basic	1	1
Aliphatic, N-1	0	0
Aromatic, N-2	18	6
Aromatic, N-3	1	0
Oxygenated, N-4	3	2
Oxygenated, N-5	< 1	< 1
Sum	28 (48%)	11 (30%)
Combined fractions	28 (48%)	14 (39%)

[a] Day samples collected at site A, street level, in February, 1979.

compounds (PNA). The compounds responsible for the mutagenicity in the acidic and the N-4 fractions have not been identified.

Analysis of PAH

The gas chromatograms of samples from sites C and D allowed quantification of 33 different PAH compounds. Table 2 shows the total concentration of PAH, together with the concentrations of pyrene and B(a)P. The concentration of PAH in particulate matter from the city air was higher in the winter than in the spring (as was its mutagenicity). The PAH concentrations in samples from sites C and D were similar.

DISCUSSION

The contribution from traffic to the mutagenicity of the air at street level appeared to be substantial. The mutagenicity of daytime samples at street level in the presence of S-9 was 4 to 20 times higher than for the corresponding samples from roof level or

Table 2. Concentration of PAH in Airborne Particulate Matter

		Concentration of PAH (ng/m^3)		
Sampling Site	Date of Sampling	Pyrene	B(a)P	∑PAH (33 comp.)
C	1978 February 20	66.0	12.0	414
	" 24	16.0	4.1	127
	" 28	3.9	2.1	72
	March 05	1.2	0.6	45
	April 04	2.1	0.9	17
	" 18	1.5	1.0	14
D	1978 February 20	40.0	7.9	319
	March 05	1.9	1.0	51
	April 04	3.5	1.4	29
C	1979 February 14	71.0	11.0	355
	" 20	5.5	3.7	60
	March 04	1.5	0.1	7
	" 10	1.1	0.6	14

from site B. Furthermore, the mutagenicity at street level varied with traffic frequency (i.e., day vs. night), whereas activity of samples from roof level and site B showed no such variations.

The mutagenicity of street samples was enhanced by the presence of liver microsomes, indicating a greater contribution from gasoline engine exhaust than from diesel exhaust. Exhaust from diesel engines is more mutagenic in the Ames test than is exhaust from gasoline engines. However, the activity of diesel exhaust is lower in the presence of S-9, while the opposite is true for gasoline-engine exhaust (Löfroth, 1979). It has been suggested that nitro-substituted PAH contributes to the mutagenic activity of exhaust from traffic (Wang, 1978). Most of these compounds are direct-acting mutagens in the Ames test.

All mutagenic activity at street level was associated with particles less than 2.7 µm in diameter. Furthermore, only traces of PAH were found in particles greater than 2.7 µm (Alfheim et al., 1977). This agrees with cascade impactor measurements made in Belgium of the size distribution of 60 organic pollutants. Van Vaeck and Van Cauwenberghe (1978) showed that 95 to 98% of the PAH, as well as some heterocyclic polyaromatics (PNA), are found in particles less than 3 µm in diameter. Most carbocyclic acids and aliphatic hydrocarbons (90%) are also associated with particles less than 3 µm.

Fractionation of extracts from particulate matter at street level showed that most of the mutagenic activity recovered was due to PNA. A similar investigation based on roof sampling in Japan showed a somewhat different result (Teranishi et al., 1978). In this study, the mutagenicity was evenly distributed among the acidic, aromatic, and oxygenated fractions (~15% in each fraction), with slight activity in the basic fraction. Of the original activity, 76% was recovered after the fractions were combined. Fractionation of particulate matter of roof samples in Stockholm (Löfroth, 1979) gave a distribution of mutagenicity similar to that reported from Japan. The recovery in our experiments was somewhat lower, possibly because the components of particulate matter collected at street level were more labile. Earlier investigations, in which particulate matter from a heavy traffic area was fractionated and tested for carcinogenicity through skin-painting experiments on mice (Wynder and Hoffman, 1965), showed that the aromatic fraction was associated with carcinogenic properties, while the acidic and oxygenated fractions were associated with promoter effects.

The mutagenicity of roof-level samples (A, C, and D) and samples from site B probably has main sources other than traffic. Mutagenicity and PAH concentrations were both higher in the February samples than in the spring samples, which is most likely explained by the higher use of residential-heating fuels during the winter months in Oslo. The average temperatures during the sampling period were -8.3°C for February, -0.9°C for March, and +3°C for April. Using measurements at street level, we found that the amount of B(a)P could only account for ~1% of the mutagenic activity in day samples and up to 4% in night samples. Data for extracts of roof-level samples from other cities show that the mutagenicity of urban air (revertants per cubic meter) was of the same magnitude in Oslo as in Stockholm (Löfroth, 1979) and Los Angeles (Pitts et al., 1977).

Meteorological conditions may also contribute to seasonal differences in mutagenicity levels and cause daily variations in the mutagenicity of winter samples. The mutagenicity (revertants per cubic meter of air) was highest on cold, clear weekdays with little wind and stagnant air.

Daily measurements of SO_2 and soot in the air have been made at these same sampling sites by the Oslo City Department of Health. The most mutagenic samples from each sampling site also had the highest concentrations of SO_2, soot, and PAH at that site.

However, while mutagenicity (revertants per cubic meter of air) and PAH concentrations were approximately equal for the two sampling sites, the values for SO_2 and soot were nearly twice as high at site C as at site D. Contributions from different pollution sources may explain why SO_2 and soot concentrations were higher at site C than at site D, while mutagenicity and PAH concentrations were equal.

Mutagenic activity in strain TA98 (revertants per cubic milligram of particulate matter) was high on days with a low total concentration of particles in the air--mainly days with rain and snow. The mutagenicity was especially high at location D on such days. Variations in the amount of nonmutagenic particles will greatly influence the mutagenicity (revertants per milligram of particles). Currently, we consider revertants per cubic meter of air to better express the mutagenic potential of the air samples. However, this subject requires further investigation.

For most samples from the rural site in southern Norway, mutagenicity either was the same with and without metabolic activation or was higher with it. The activity with metabolic activation might be explained by the presence of PAH compounds in the samples. Such compounds have previously been demonstrated in long-range-transported aerosols collected at this site (Lunde and Bjϕrseth, 1977), and their amount have been shown to vary with the origin of the air masses in the same way as the mutagenic effect does. Like the mutagenicity, the concentration of PAH at site E also was 5 to 10% of that found in samples from Oslo. At site F, the PAH concentrations were up to 1% of the corresponding Oslo values.

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DETECTION OF GENETICALLY TOXIC METALS BY A MICROTITER MICROBIAL DNA REPAIR ASSAY

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INTRODUCTION

For some years, our laboratory has been involved in assessing the genetically toxic effects of inorganic chemicals found in the environment near mining and smelting operations (Tindall et al., 1978; Warren et al., 1979) and of metal-containing pesticides used in Montana (Warren et al., 1976). Although many inorganic species are known or suspected carcinogens or are genetically active in many test systems (Flessel et al., 1979; Sunderman, 1978), most existing short-term biological screening methods are unsuitable for use with this class of suspected carcinogen. Only two systems, the *Bacillus subtilus rec* assay (Nishioka, 1975; Kanematsu et al., 1980) and an *in vitro* DNA synthesis fidelity assay (Loeb et al., 1979), have been useful for a large number of inorganic chemicals. Mutagenicity of some inorganic chemicals has been demonstrated in a CHO/HGPRT assay (Hsie et al., 1979) with considerable technical difficulty due to the general toxicity of inorganic chemicals. Green and Muriel (1976) have used a repair-deficient series of *Escherichia coli* B strains to detect mutagenicity of some chromate salts; also by this method, they found that nickel chloride salts do not cause differential lethality.

Recent discoveries about the relationship of DNA repair functions to mutagenesis in bacteria (Witkin, 1976; Kimball, 1978) and of error-prone repair mechanisms such as inducible SOS repair and another excision repair mechanism (Hanawalt et al., 1979), provide reasons for using batteries of repair-deficient organisms in a screening system, rather than using a single repair-deficient strain, such as *pol A* (Hyman et al., 1980). The pleiotropy of *rec*$^-$ mutants is a major problem in using only one double-*rec*-deficient

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strain, such as Bacillus subtilis M45, for the assay. Such rec⁻ mutants exhibit permeability changes, possibly due to cell-wall and membrane-surface defects, as in rec A (Tomizawa and Ogawa, 1968). Differences in inhibitory effects of test chemicals might be due to differences in their rates of penetration into the repair-defective strain as compared with wild type rather than to differences in DNA repair capacity.

To rapidly screen large numbers of samples, we have developed a microbial repair assay using a series of singly- and multiply-mutant DNA-repair-deficient strains in an E. coli K12 background. The mutational repair defects of the K12 series have been studied in much more detail, both genetically and biochemically, than have those of E. coli B or B. subtilis (Witkin, 1976; Kimball, 1978; Hanawalt et al., 1979). The strains are stable in culture and easy to grow, and they carry several biochemical markers that permit genetic manipulation for strain construction, and possibly mutagenesis assays, in the same repair strains. A number of metal salts and metal salt compounds have been tested in the K12 system (Warren et al., in press a, b; MS). Our results correlate well with mutagenesis as assayed by the Ames test. This paper describes in detail the repair assay and its use to assay metal-containing samples.

METHODS

Construction and Testing of Strains

The derivation of the repair-deficient E. coli bacterial strains is diagrammed in Figure 1. In each case, isogenicity of the repair defectives with the wild type AB1157 was optimized, to minimize mutational effects other than on DNA repair. For discussions of repair defects and phenotypes see Kimball (1978), Hanawalt et al. (1979), and Witkin (1976).

The bacteria are stored at room temperature in Lederberg stabs of nutrient broth (Difco) and have remained viable for at least three years. Each month, working slants are made from single colonies and stored at room temperature. Bacteria from these single colonies are streaked onto minimal medium plus the amino acids required by AB1157 and onto minimal medium alone, to verify the strains' nutritional requirements. Every two weeks, single colonies are isolated from the working slants to check the repair characteristics of the strains. Two colonies of each strain are isolated and inoculated into Mueller-Hinton (MH) broth (5 ml), and cultures are grown overnight at 37°C. Ultraviolet-light sensitivity of each culture is checked by the rapid method of Greenberg (1967) as follows: A streak of each strain is placed by capillary pipette on each of seven MH agar plates. Plates are

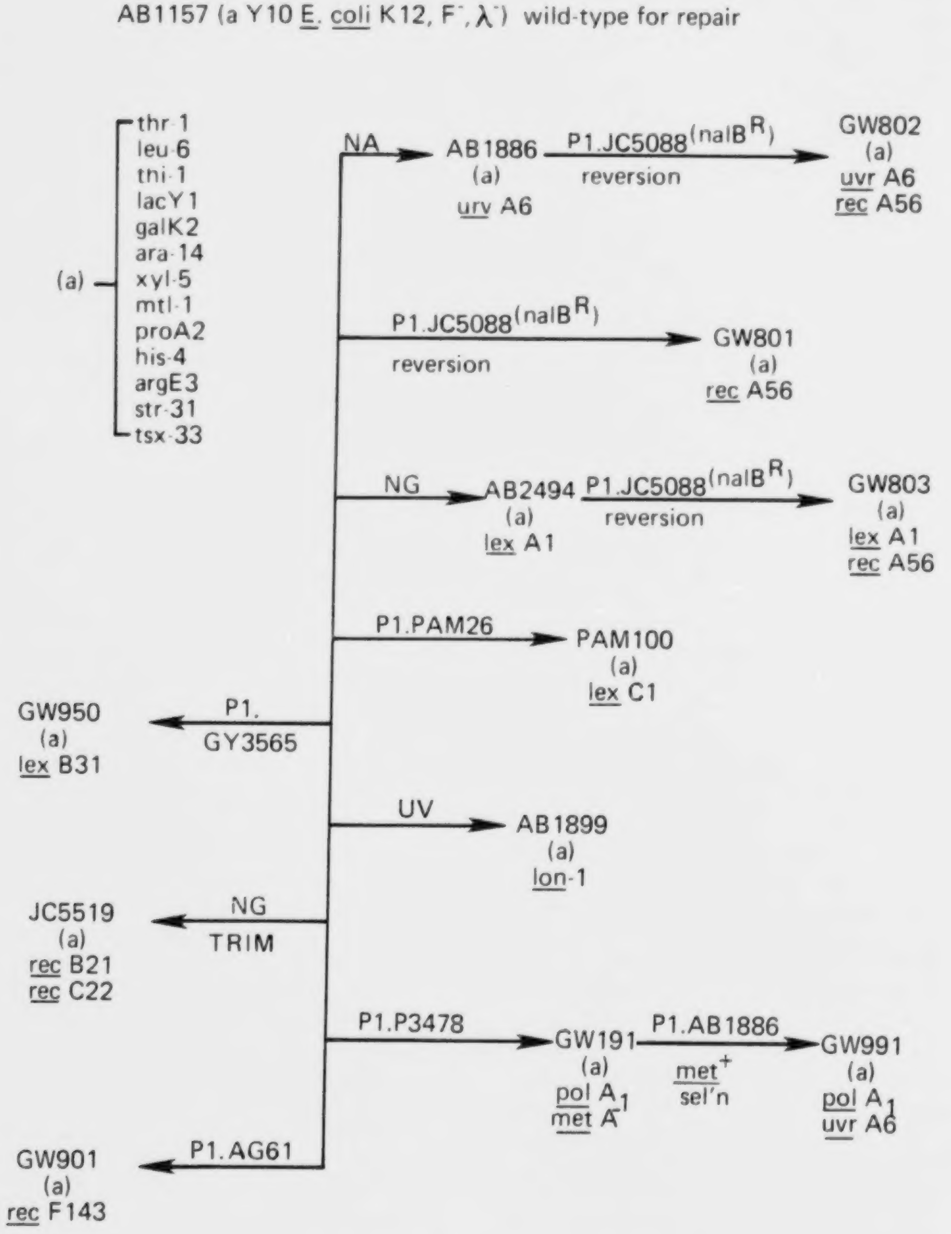
AB1157 (a Y10 E. coli K12, F⁻, λ⁻) wild-type for repair
(a)
thr-1
leu-6
thi-1
lacY1
galK2
ara-14
xyl-5
mtl-1
proA2
his-4
argE3
str-31
tsx-33
NA
AB1886 (a) urv A6
P1.JC5088(nalB^R) reversion
GW802 (a) uvr A6 rec A56
P1.JC5088(nalB^R) reversion
GW801 (a) rec A56
NG
AB2494 (a) lex A1
P1.JC5088(nalB^R) reversion
GW803 (a) lex A1 rec A56
P1.PAM26
PAM100 (a) lex C1
P1. GY3565
GW950 (a) lex B31
UV
AB1899 (a) lon-1
NG TRIM
JC5519 (a) rec B21 rec C22
P1.P3478
GW191 (a) pol A1 met A⁻
P1.AB1886 met⁺ sel'n
GW991 (a) pol A1 uvr A6
P1.AG61
GW901 (a) rec F143

Figure 1. Construction of a series of E. coli K12 repair-deficient strains from strain AB1157 (wild-type for repair).

immediately irradiated with known fluences of 254-nm light (for example, 0, 20, 50, 100, 200, 300, and 400 ergs/mm^2) and then incubated in the dark at 37°C. Results such as those shown in Table 1 can be determined after incubation for 18 h. Any culture not giving the expected result is rejected, and a new colony of that strain is picked and tested. Acceptable cultures are labelled for use and stored. Broth cultures may be used for testing for up to two weeks if stored at 2°C. Several of the repair-deficient strains are still being evaluated as testers, and those with the most useful traits will be chosen. An example is the *lex* C mutant strain, PAM100, which is uniquely sensitive to nickel compounds.

We are constructing a second identical isogenic series of strains containing an *rfa* (deep rough) mutant locus. This series will be tested later this year. A series of repair-deficient strains containing the ochre mutant *trp*$^-$ locus from WP2 and one strain containing two of the Yanofsky *trp*$^-$ series (Yanofsky, 1963) for detecting frameshifts and base pair substitutions are under construction.

ASSAYS

Initially, we developed a sensitivity disc assay in which zones of inhibition caused by inorganics were compared between each repair-deficient strain and the wild-type (repair-proficient) strain. Such a method allowed differential inhibition (in many instances, lethality) to be grossly quantitated. DNA damage of some sort should have been the cause, because the only differences between strains were in repair capacities.

The sensitivity disc assay was done by spreading 0.1 ml of each broth culture directly on a complete-medium agar plate. Sterile discs of paper were impregnated with a known concentration of test agent. Three of these discs were placed on each freshly spread agar surface, and plates were run in triplicate. Several different media were tried initially, in an effort to maxmimize zone size differences. For a group of salts of known metal carcinogens (chromium, cadmium, mercury, cobalt, and arsenic), the largest differences were obtained on solid medium containing 10 g/l agar and MH broth (Difco). When 0.2% glucose was added, chromate compounds gave a much clearer and larger differential response, indicating a glucose requirement for rapid bacterial growth. Addition of 0.5% sodium chloride increased the inhibitory effects of cadmium and cobalt. The standard medium now used is MH plus glucose and salt. The effects of these additions to MH medium are shown in Table 2 as differences in zones of inhibition (mm). Differences greater than 5 mm were significant at the 95% level (Tindall, 1977).

Table 1. Verification of Repair Deficiencies by Ultraviolet Irradiation

		Growth in Streaks on MH Plates After Irradiation with 254-nm Light[b]						
		Fluences (ergs/mm^2)						
Strain	Repair Defect[a]	0	20	50	100	200	300	400
AB1157	wild type	+++	+++	+++	+++	+++	+++	+++
AB1886	uvr A6	+++	++	- (1)	- (20)	- (0)	- (1)	- (2)
AB1899	lon-1	+++	+++	+++	+++	+++	+++	- (8)
AB2494	lex A1	+++	+++	+	+-	- (15)	- (0)	- (0)
GW801	rec A56	++	+-	- (>200)	- (35)	- (0)	- (0)	- (0)
PAM100	lex C1	++	+++	+-	-+	- (20)	- (0)	- (0)
GW802	uvr A6, rec A56	+++	- (3)	- (0)	- (0)	- (0)	- (0)	- (0)
GW803	lex A1, rec A56	+++	- (>200)	- (45)	- (8)	- (3)	- (0)	- (0)
GW191	pol A1	+++	+++	+++	+++	+-	-+	- (36)

[a] Actual kill curves can be determined if necessary.

[b] +++ = maximal growth; ++ = noticeably less dense streak; + = thin streak; +- = visible lethality; -+ = spotty but >500 colonies; - = kill (no. of colonies counted).

Table 2. Effects of Additions to Growth Medium on Differential Inhibition by Metals: Difference from Strain AB1157 (Wild Type) in Zone Diameter (mm)

	Carcinogenic Metal Salts															
	K_2CrO_4[a]				Na_2TeO_3[b]				$BeSO_4$[c]				$KSbO_4(C_4H_4O_6)\cdot 1/2H_2O$[d]			
Medium[e]:	MH	MHG	MHS	MHGS	MH	MHG	MHS	MHGS	MH	MHG	MHS	MHGS	MH	MHG	MHS	MHGS
Strain																
AB1886	0	-10	3	-4	5	5	3	4	0	0	0	0	2	5	4	2
GW801	2	3	3	6	-4	1	-2	0	0	0	0	0	-1	7	6	4
AB2494	-4	-14	-2	2	12	16	16	8	0	1	0	0	-2	6	1	1
GW802	1	10	3	12	-2	3	0	2	10	10	8	0	-1	11	4	4
GW803	1	4	3	5	16	6	3	8	0	0	0	0	-7	0	2	1
GW191	0	3	2	9	10	14	15	12	0	0	1	0	-6	3	-3	-1

[a] 0.10 M in H_2O, 10 µl/disc.
[b] 0.01 M in H_2O, 15 µl/disc.
[c] 0.10 M in H_2O, 20 µl/disc.
[d] 0.10 M in H_2O, 20 µl/disc.

[e] MH = Mueller-Hinton agar (Difco).
MHG = MH agar + 0.1% glucose.
MHS = MH agar + 0.05% NaCl.
MHGS = MH agar + glucose + NaCl.

Kanematsu et al. (1980) used a cold incubation period after spreading and disc placement to enhance the inhibition by test metals in the B. subtilis rec assay (this allows the test substance to diffuse before bacterial growth starts). We have observed this effect with most chemicals, although some (mostly organic compounds) have shown weaker responses after cold incubation than without it.

Because migration through an agar medium is affected by the depth and viscosity of the agar, temperature, charge, and pH, and because all of these parameters are subject to laboratory variation, we searched for a more reproducible and sensitive method. The disc repair assay required high levels of test agent and was not quantitative, due to solubility problems. McCarroll et al. (1979) have developed a microtiter assay system to study effects of organic toxicants using repair-defective strains of E. coli B obtained from Green and Muriel (see Green and Muriel, 1976; McCarroll et al., 1980a, b). We have adapted this system to the repair assay with E. coli K12. Microtiter techniques (Cooke Engineering, 1972) can be used to quantitatively measure inhibition of growth (as in antibiotic testing) and also to quantitatively measure lethality, since colonies can be counted in each well at the end of a treatment period (McCarroll et al., 1980b). Mutagenesis testing also can be done from the same microtiter plate wells, thus generating actual mutation frequencies (mutants per survivor). Several loci could be monitored for mutagenesis at the same time.

A microtiter plate set up for an eight-strain test is shown in Figure 2. Initially, 0.05 ml of MH broth is added to each well of the microtiter plate, using a Tridak Stepper, 3-ml syringe, or Gilson Repetman. Rows 1 and 12 receive a double dose. Rows A through H will each receive one of the strains of bacteria, with A receiving the wild type. Row 1 will contain only bacteria and medium, to serve as the cell control. Row 2 receives 0.05 ml of the test agent in solution. For most inorganic toxicants, a convenient initial concentration is 2 mg/ml in water, if possible (or dimethylsulfoxide if necessary). The test agent is added to row 2 with a 1-ml Tridak stepper. Serial dilutions are then performed from row 3 through row 11, using microtiter diluters (Cooke), which hold 0.05 ml and do automatic serial dilutions. The final dilutions of mutagen are shown in Figure 2 and range from 1:4 (row 2) to 1:2048 (row 11). Row 12 is left uninoculated as a sterility control for medium, mutagen, and solvent. Having been checked and stored as described above, each of the bacterial cultures is diluted in MH to a final viable count of ~ 1 x 10^6 colony forming units (CFU)/ml, for optimal sensitivity in microtiter. Because several of the repair-deficient strains (especially the multi-mutants; see Table 1) do not reach the same cell density overnight as does the wild type, the appropriate

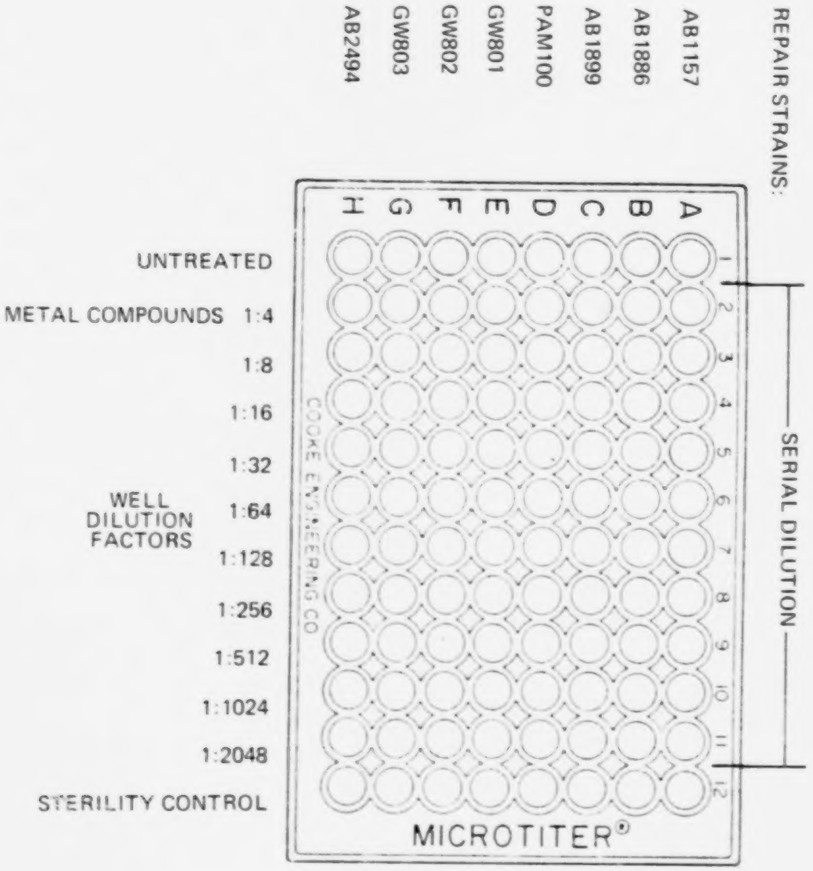
REPAIR STRAINS:
AB1157
AB1886
AB1899
PAM100
GW801
GW802
GW803
AB2494
A
B
C
D
E
F
G
H
1
2
3
4
5
6
7
8
9
10
11
12
UNTREATED
METAL COMPOUNDS 1:4
1:8
1:16
1:32
1:64
1:128
1:256
1:512
1:1024
1:2048
STERILITY CONTROL
WELL DILUTION FACTORS
SERIAL DILUTION
COOKE ENGINEERING CO
MICROTITER®

Figure 2. Sample microtiter plate.

dilution must be established for each strain. Bacterial culture dilutions should be discarded daily; they may be used all day if kept on ice. With a 1-ml Tridak stepper, 0.05 ml of bacterial culture dilution is added to each well of the appropriate row for that strain.

Initial concentrations of test agents can be varied, within limits of solubility, to test for effects throughout a very large concentration range. Plates are sealed with tape to retard evaporation. Sterile lids can be used for short-term treatments, but incubation for over four hours results in significant fluid loss by evaporation. The standard incubation time is 24 h. Some tested compounds initially inhibit growth, but then either resistant organisms overgrow the wells or the test agent is metabolized and becomes ineffective. For such agents, a shorter incubation time can be used.

Mammalian microsomal fractions can be used for activation in the repair assay. Positive controls such as 2-aminoanthracene show good responses with either of two methods of addition of rat liver homogenate (S-9). The S-9 mix is prepared according to Ames et al. (1975), and 0.05 ml is added with the mutagen to wells in row 2, in place of the initial 0.05 ml of MH broth. Both substances are simultaneously serially diluted through the wells. Alternatively, one may add 0.05 ml of S-9 mix in place of MH broth to each well in the plate and then dilute only the test mutagen. Throughout either procedure, all plates are kept on ice. For all positive controls we have studied, the former method results in more differential lethality than the latter. After incubation, the plates are read visually, using a microtiter mirror. For each of the lettered rows, the totally inhibited well with the lowest test-agent concentration is determined visually and recorded. From the dilution factor for that well and the known concentration of test agent, a minimal inhibitory concentration (MIC) can be determined for each strain. Differential inhibition is given as the factor of increase in MIC over that of the wild-type strain, in this case AB1157 (i.e., MIC for repair-deficient strain/MIC for repair-proficient strain).

RESULTS

Table 3 compares the results of the microtiter repair assay and the disk assay, using eight strains of E. coli and ten rhodium complexes recently synthesized by Abbott (Warren et al., in press a).

In the optimal test, an inhibitory test-agent concentration for the wild-type bacteria would be determined. In practice, this is not possible for all agents, because the wild type often is resistant to all concentrations within the range of solubility of the test agent. In such cases, the highest concentration that allows determination of an MIC for the most sensitive strain has been used (less than 11 wells inhibited). The factor of increase in MIC is therefore underestimated.

A series of substitutionally inert rhodium complexes was used to demonstrate the utility of the method described here, not only for detecting DNA damage but also as a screening test preceding the Ames reversion assay (Warren et al., in press a). Dose responsiveness was predictable, and as the dose was doubled, the MIC also doubled, until the entire plate was killed. The factor of increase in MIC for any one test agent did not change once a lethal

Table 3. Comparison of Microtiter and Disc Repair Assays, Using Rhodium Complexes

	Inhibition of Strains Tested by the Disc (D)[a] and Microtiter (M)[b] Methods											
	AB1886 uvr A6		GW801 rec A56		AB2494 lex A1		GW802 uvr A6 rec A56		GW803 lex A1 rec A56		AB1899 lon-1	
Complex	D	M	D	M	D	M	D	M	D	M	D	M
$[Rh(Pyr)_4Br_2]Br$	18.0	16	23.4	32	12.7	0	24.4	512	22.0	8	13.4	4
$[Rh(CH_3CN)_3Cl_3]$	9.3	0	7.3	4	2.0	0	12.0	12	8.7	4	2.7	0
$[Rh(Bipy)_2Cl_2]Cl$	7.5	0	0	0	0	0	16.5	16	0	0	0	0
$[Rh(3Pic)_4Cl_2]Cl$	15.3	16	23.0	2	12.7	0	28.3	128	20.0	0	14.7	16
$[Rh(Pyr)_4Cl_2]Cl$	23.0	8	22.6	0	7.5	0	27.0	256	235.0	0	123.0	0
$[Rh(Phen)_2Cl_2]Cl$	9.0	0	2.0	0	0	0	7.0	64	1.5	0	0	0
$[Rh(en)_2]Cl_2$	0	0	0	0	0	0	7.6	16	0	0	0	0
$RhCl_3(H_2O)_3$	6.6	0	3.3	0	0.5	0	8.6	8	4.3	0	2.0	0
$[Rh(NH_3)_4Cl_2]Cl$	0	0	0	0	0	0	12.0	16	1.3	0	0	0
$[Rh(Trien)Cl_2]Cl$	1.3	0	12.3	0	0.5	0	23.3	64	16.3	0	0.6	2

[a] For test agents at 20 mg/ml H_2O, 20 µl/disc; means of results from 6 discs; expressed as difference in diameter from wild type (mm).

[b] For test agents at 2 mg/ml H_2O, without S-9; means of results from duplicate plates; expressed as factor of increase in MIC over that of wild type.

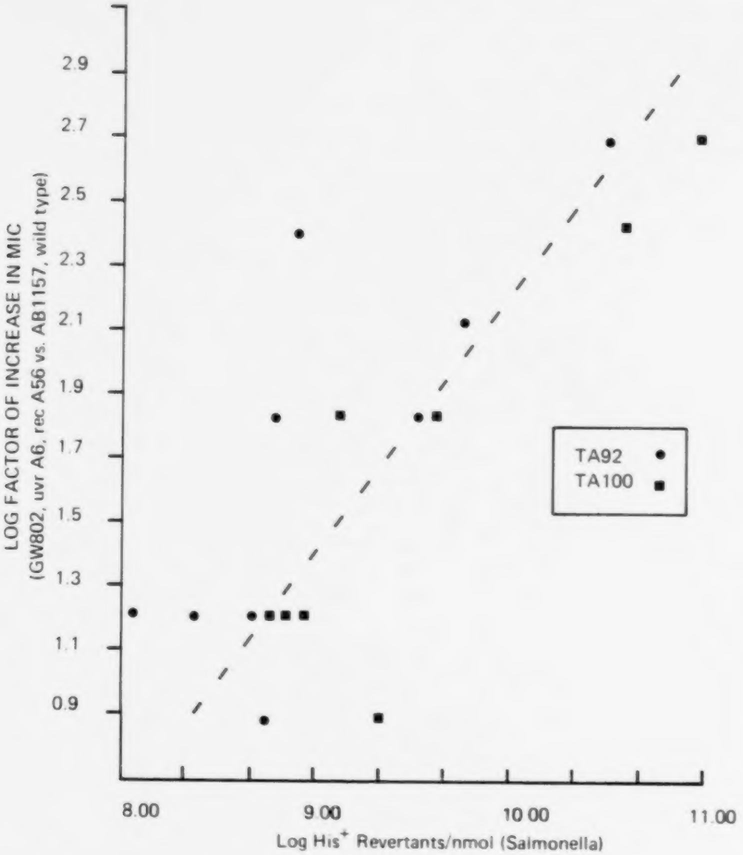

Figure 3. Correlation between sensitivity of the microtiter repair assay (using strain GW802, uvr A6, rec A56) and mutagenicity (strains TA92 and TA100) in the Ames test for a group of rhodium complexes.

concentration was reached in the wild-type strain. A comparison between the results of the microtiter repair assay and the Ames test for these compounds is given in Figure 3. The coefficient of correlation between the results of the two tests was 0.92, indicating agreement. No Ames-positive test agents in a series of 23 rhodium complexes were missed by the repair assay, while one repair-positive agent (very weak) was Ames negative.

An S-9 dose response in the microtiter repair assay with an organic herbicide, diallate, is shown in Figure 4. Diallate, an indirect-acting mutagen, was used because this compound is small

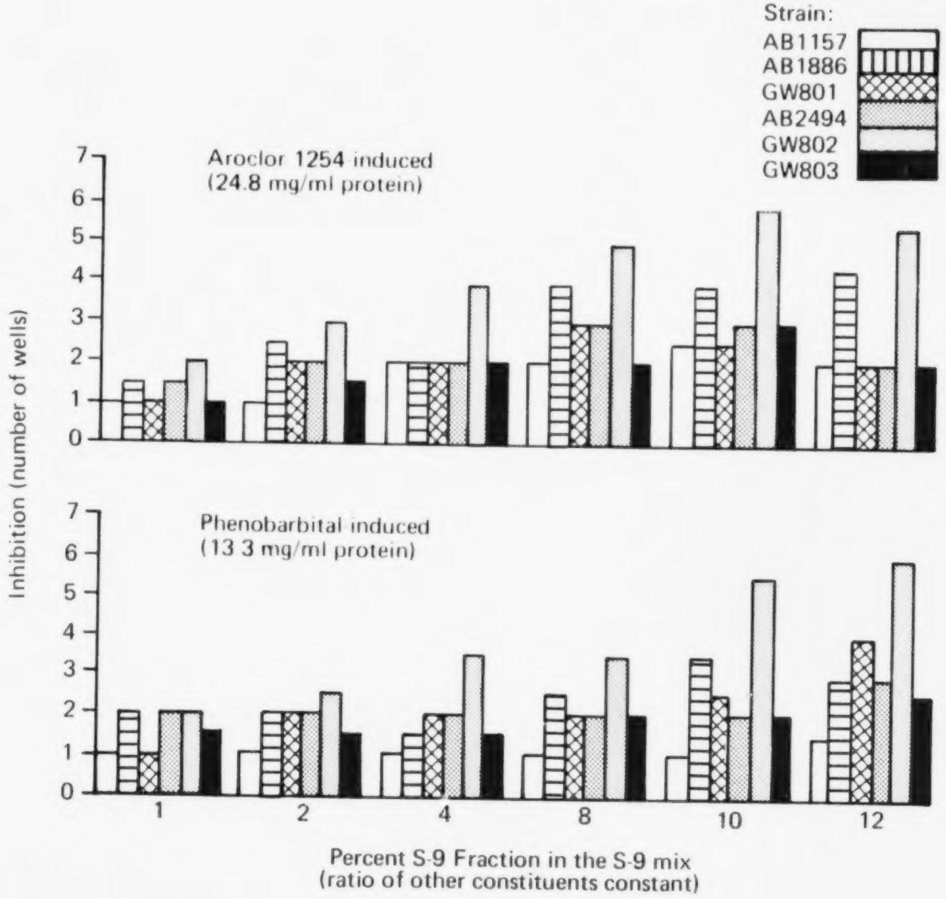

Figure 4. Enzyme activation of diallate in a microtiter repair assay.

and should have no difficulty penetrating cell walls. The results were related to the enzyme dose. Also, the type of inducer used affected the response just as it does in a mutagenesis assay. We have previously noted that a higher concentration of protein is required of phenobarbital-induced enzyme than of Aroclor-1254-induced S-9 for diallate mutagenicity in the Ames test. While the inhibitory activity of several metal salts was reduced by S-9 (zinc, palladium, mercury, antimony, arsenic, selenium, nickel, manganese, and cadmium), we have seen only one in which S-9 increases genetic toxicity (tellurium)(Rogers and Warren, unpublished data).

This system is designed for testing complex mixtures. It has been used to document the differential lethality of a sample of smelter rafter dust, air filter particulate extracts, and urine concentrates (Warren et al., 1979).

DISCUSSION

The microtiter repair assay techniques described here employ standard microtiter equipment and can be automated. A scanning device is available for reading optical density directly from the plates for growth determinations. Various dyes can be used to indicate growth (e.g., bromophenol blue)(Green and Muriel, 1976). In cases of agents which kill cells but cause filamentation of some strains, plate counting may be required, because filamentation increases optical density without an increase in the number of viable cells. This is particularly likely with strains AB1899 and PAM100, both of which filament easily and in response to any chemical insult.

Strains of *E. coli* have been constructed with mutations to block incision (*uvr* A, B, C) and to partially block excision (*pol* A_1, *rec* B, C), resynthesis and conditioned responses (*rec* A, *lex* A), and recombination (*rec* A, *rec* F). Mutagenesis might be caused by less-well-known repair mechanisms that could be unmasked if the more rapid error-free repair were removed by mutation and post-replicative repair were incapacitated as well. The strain GW802 has these defects and is easily the most sensitive strain in the battery at this time. There is some evidence that an SOS-like conditioned response system exists in mammals and may be responsible for tumor induction by some mutagens (Radman, 1977). Many of the metal mutagens and all of the substitutionally inert transition metal complexes we have tested require the pKM101 plasmid in *Salmonella* for mutagenesis and do not require a functional *uvr* system. Venturini and Monti-Bragadin (1978) found that no mutation is caused by platinum without the *lex* function in *E. coli*, but that *lex*$^+$ *E. coli* can be mutated without carrying pKM101. Therefore, these strains are suitable for metal-ion mutagenesis without the pKM101 repair system. Because many mutagens can function as curing agents, a system that does not require a plasmid would seem to be advantageous.

If an agent's lethal and mutagenic effects on each bacterial strain are known, then the general type of DNA damage caused by that agent can be predicted. For instance, *uvr*$^-$ strains cannot recognize DNA helix distortions; agents differentially lethal to *uvr*$^-$ strains but not mutagenic are most likely to be strand cross-linkers (Murray, 1979). Green and Muriel (1976) give five classes of damaging agents and note that some mutagens fit in more than one category.

It has also been shown that repair assays correlate better than do mutagenesis or prophage induction with anti-tumor activity of alkylating agents (Tamaro et al., 1977), indicating another useful screening capacity for the microtiter repair system.

CONCLUSIONS

The microtiter technique is rapid, cost effective, and sensitive and requires very little sample. The bacterial strains are easily grown and checked and provide an accurate screening test for both organic and inorganic genetically toxic chemicals and complex mixtures, regardless of their activity in mutagenesis systems. This test is a very useful prescreen for mutagenesis assays.

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A CULTURE SYSTEM FOR THE DIRECT EXPOSURE OF MAMMALIAN CELLS TO AIRBORNE POLLUTANTS

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INTRODUCTION

Most airborne pollutants first enter the body through the respiratory tract. In mammals and most other air-breathers, mechanisms have evolved to deal with these pollutants, especially those of a particulate nature. In recent times, however, air pollutant gases have been introduced into the environment at higher concentrations than before. Well-known examples are ozone (O_3) and oxides of nitrogen (NO_2, NO). Other gaseous pollutants whose effects are not so well known include short-lived, highly reactive species produced photochemically in the urban air mixture called smog. Peroxyacetyl nitrate (PAN) is one example (Stephens, 1969).

There has not been sufficient time for animals to evolve resistance to atmospheric oxidant gases. This lack of inherent resistance can be shown dramatically in rats exposed to 0.8 ppm of O_3 in air. At rest, the rats can tolerate this concentration for several hours, but if they are forced to exercise (i.e. on a treadmill) they quickly succumb to lung edema and hemorrhage (R.F. Phalen, University of California, Irvine, personal communication, 1980). Rats can be acclimatized to O_3 by repeated exposure at rest, so that they can resist the effects of O_3 when forced to exercise. Thus, substantial adaptational changes must have occurred in the lung. Studies of various enzymes in lungs of rats exposed to O_3 and NO_2 also have shown alterations (Chow et al., 1976). It can be supposed that similar effects occur in the human lung, since oxidant gas concentrations in some areas frequently approach, and even exceed, 1 ppm.

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The cell culture and exposure system described in this report represents an in vitro approach to the study of the initial interactions between oxidant gases and respiratory cells. The goals of the present studies were to document some of the cytotoxic, biochemical, and cytogenetic effects on mammalian cells of exposure to low concentrations of O_3 and NO_2. Certain features of this system allow the exposure of cell cultures to ambient polluted atmospheres in a manner resembling exposure at the surface of the respiratory epithelium. Living cells are separated from the test atmosphere only by a thin layer of nutrient medium held over the cells by capillary attraction. As this situation allows close contact between cells and the ambient atmosphere, it may be especially useful in studying the primary interactions between cells and airborne pollutants.

MATERIALS AND METHODS

Exposure System

The following features are required in a cell system for testing exposure to gaseous pollutants:

1) accurate generation and monitoring of the pollutants to be studied,

2) maintenance of the cells in close contact with the test atmosphere without allowing them to dry out,

3) exposure under biologically sterile conditions, and

4) provision for recovery of the cells for further culturing and analysis.

The polluted atmospheres were generated by the measured addition of NO_2 or O_3 to a stream of clean air. The initial exposure system consisted of one control and one experimental chamber. Results with this early system have been reported elsewhere (Samuelsen et al., 1978; Crocker et al., 1979). The system was later expanded to a total of six chambers that could be arranged as desired for control or text exposures. Figure 1 is a diagram of the system, showing interconnections for using three different gases, either individually or in combination. Atmospheres containing NO_2 or sulfur dioxide (SO_2) were generated by diluting gas from stock cylinders in a stream of clean air. Clean air was provided by treating the building air supply sequentially with Purafil, activated charcoal, and filtration through a 2-μm bacteriological filter. This treatment reduced the level of NO_2 to < 10 ppb and the O_3 level to below detectable

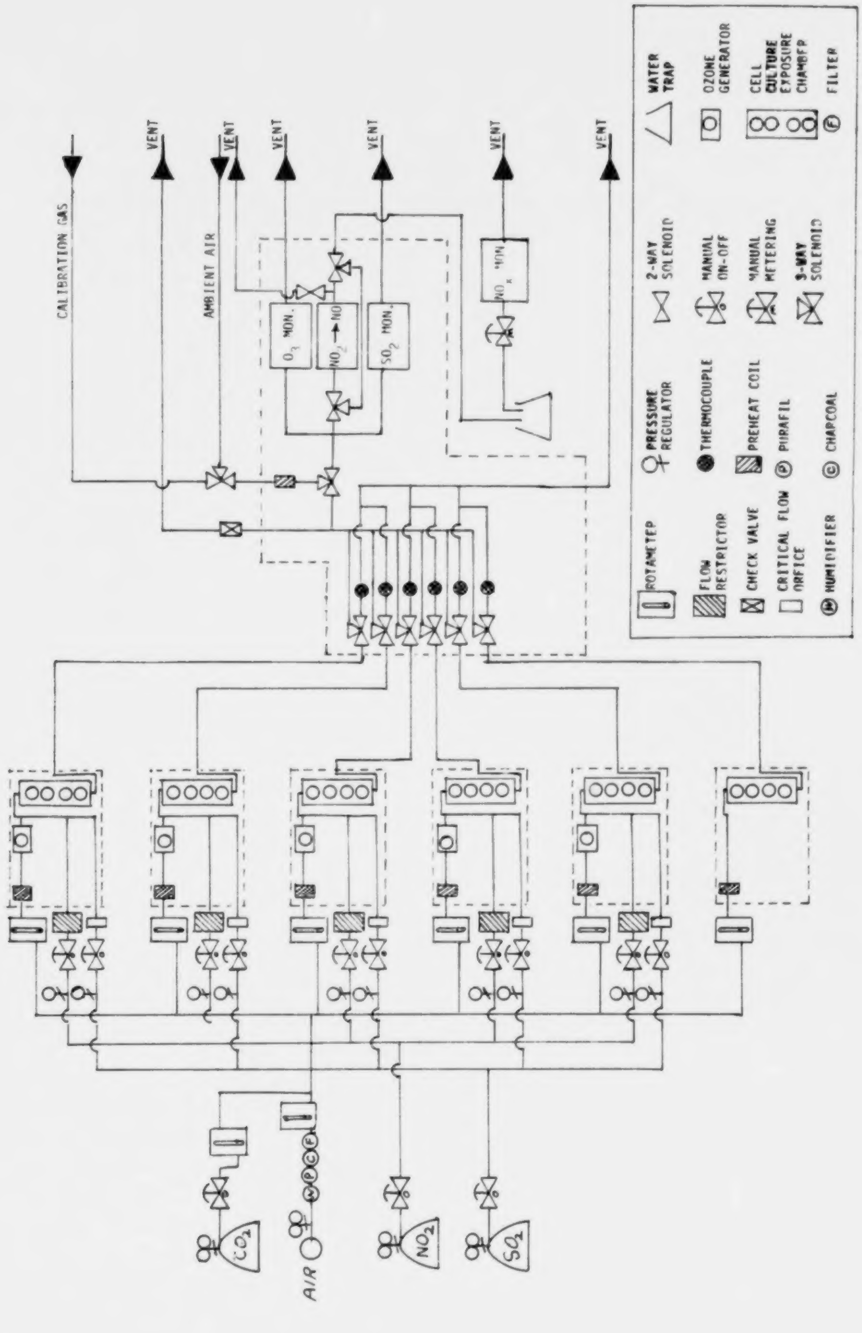
CALIBRATION GAS
AMBIENT AIR
VENT
VENT
VENT
VENT
VENT
VENT
O_3 MON.
$NO_2 \rightarrow NO$
SO_2 MON.
NO_x MON.
CO_2
AIR
NO_2
SO_2
ROTAMETER
FLOW RESTRICTOR
CHECK VALVE
CRITICAL FLOW ORFICE
HUMIDIFIER
PRESSURE REGULATOR
THERMOCOUPLE
PREHEAT COIL
PURAFIL
CHARCOAL
2-WAY SOLENOID
MANUAL ON-OFF
MANUAL METERING
3-WAY SOLENOID
WATER TRAP
OZONE GENERATOR
CELL CULTURE EXPOSURE CHAMBER
FILTER

Figure 1. The exposure system.

amounts (i.e., < 1 ppb). Because the cell culture medium was bicarbonate-buffered, carbon dioxide (CO_2) was added to the air stream to raise its concentration to 5%.

Each exposure chamber was provided with flow controls for the clean air and pollutant gases. The gases were mixed with the air stream immediately before they entered the exposure chambers. Ozone was provided by an individual generator for each exposure chamber. The chambers were enclosed in 37°C incubators. The chambers were rectangular with internal dimensions of approximately 10 x 10 x 35 cm and volumes of approximately 3.5 l. Gas flow was along the long axis of the chamber and could be adjusted up to a flow rate of 4 l/min. All tubing and fittings were either Teflon or stainless steel. The exposure chambers were lined with stainless steel foil; chambers of Lexan plastic are also available.

From the exposure chambers, the gas was carried via heated sampling lines to a bank of solenoid valves controlled by a microprocessor. The outflow from each chamber was sequentially directed to the monitoring instruments, which (except for the NO_x monitor) were enclosed in a 37°C chamber to prevent moisture condensation in the instruments. The instruments were selected to provide measurements of specific gases without interference from other gases. These instruments were a Dasibi model 1003 AH O_3 monitor and a Beckman model 952A NO_x analyzer. (A monitor for SO_2 had yet to be installed.) Output from the monitors was fed to a multichannel recorder.

Cell Culture Method

Cells of strain V-79 Chinese hamster lung fibroblasts (obtained from Dr. E.H.Y. Chu, Ann Arbor, MI) were routinely grown in Eagle's minimum essential medium supplemented with 10% fetal bovine serum. All cell culture media were from Grand Island Biological Co.

For exposure to gases, the cells were planted, either as dispersed cells or as confluent cultures, on Millipore filters (HAWP, 0.47-µm pore size) that had been thoroughly washed and autoclaved. The cell-bearing filters were then assembled into the specially designed holder shown in Figures 2 and 3. These holders were fabricated from either Lexan plastic (General Electric Co.) or stainless steel. The filters were positioned cell-side up and sealed in place with the O-rings and threaded cap. Growth medium was provided to the cells with a syringe pump connected to the fitting in the base of the holder. Medium perfusing through the filter was drawn off through a small tube in the membrane holder, shown in Figure 2 as a diagonal line at the right-hand side of the membrane holder. With this arrangement the cells were kept moist

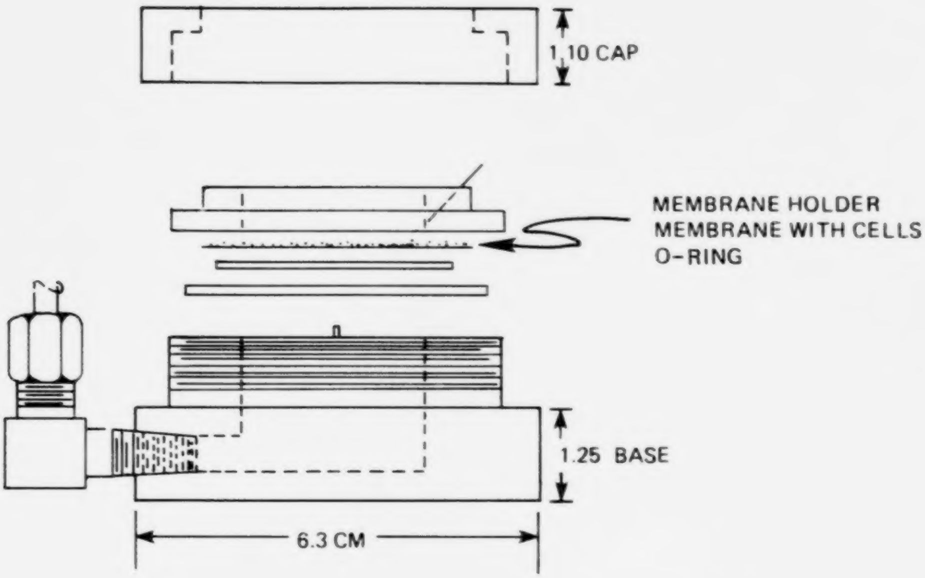
1.10 CAP
MEMBRANE HOLDER
MEMBRANE WITH CELLS
O-RING
1.25 BASE
6.3 CM

Figure 2. Diagram of a filter holder.

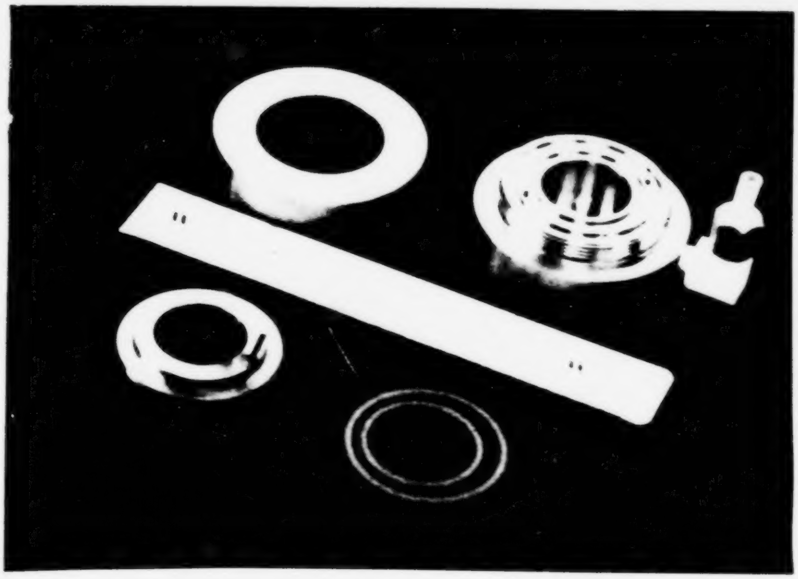

Figure 3. Photograph of a disassembled filter holder.

and continuously provided with fresh nutrient, while in nearly direct contact with the ambient atmosphere. In clean air, nearly 100% viability could be maintained for at least several hours.

Experimental Procedure

Figure 4 shows a filter with cells being placed in a holder. Before assembly, the base was filled with medium, and after assembly, the top well of the holder was filled with medium to protect the cells before placement in the exposure chambers. The holders were then placed in the exposure chambers (four per chamber). The bases of the holders were connected to the syringe pump, while the tubes for withdrawal of medium from the upper wells were connected to a peristaltic pump. The chamber door was sealed, and gas flow was initiated. When the desired pollutant concentration within the chamber had been reached and was stable, the medium overlaying the cells was drawn off with the peristaltic pump, and cell exposure was begun. The syringe pump was turned on at the same time to slowly perfuse nutrient medium through the filter. To conclude the exposure, the exposure chambers were opened, the holders removed, and the top well immediately filled with nutrient medium. Because the gas exposure levels used were of the same order of magnitude as in the ambient atmosphere (0.15 ppm NO_2; 0.05 ppm O_3), no special precautions were necessary.

Subsequent procedures depended on the nature of the experiment. To estimate colony formation by surviving cells, the filters, previously seeded with an appropriate number of dispersed single cells, were transferred directly to petri dishes containing nutrient medium and incubated for 7 to 10 days to permit colony development. The cells could then be harvested from the filters with trypsin and subcultured for further study, such as for mutagenesis or chromosomal effects.

Measurement of Effects of O_3 and NO_2 on DNA Replication

The procedures for this study were adapted from those described by Painter (1977). Cultures of V-79 cells were grown for 48 h with carbon-14-labeled thymidine (^{14}C-TdR) at 0.01 µCi/ml (50 mCi/mmol). The cells were then planted on filters and exposed to O_3, NO_2, or clean air at the concentrations and for the times indicated in the Results section, below. After the gas exposure, the filters were returned to nutrient medium; at intervals, sample cultures were labeled for 10 min with tritiated thymidine (^{3}H-TdR; 5 µCi/ml, 60 Ci/mmol). After this labeling, the filters were fixed in ice-cold 5% trichloroacetic acid, washed several times with 70% ethanol, and air dried. The radioactivity associated with the filters was measured by scintillation counting, and the ratio of

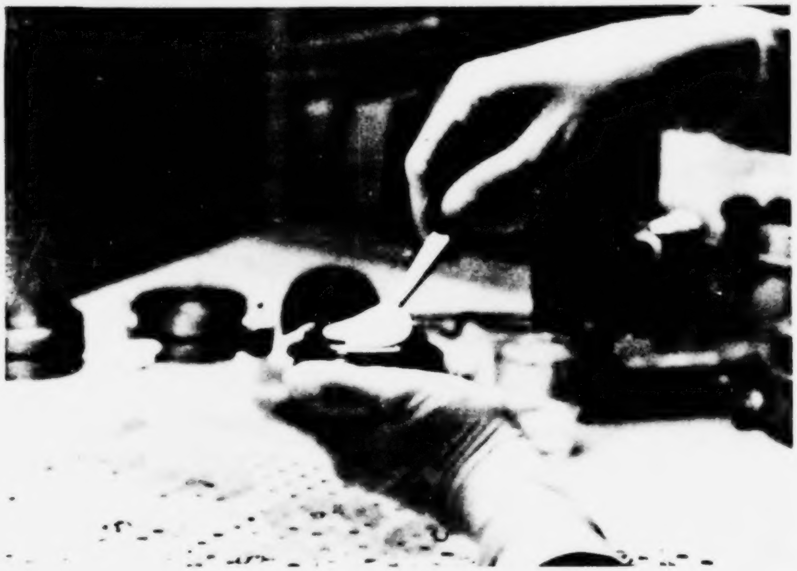

Figure 4. The filter with cells being placed in the holder.

^{3}H dpm:^{14}C dpm was calculated to give an index of the rate of DNA synthesis at the time of labeling with ^{3}H-TdR.

Measurement of Cytotoxic Effects of O_3 and NO_2

Strain V-79 cells were seeded into filters as dispersed single cells and allowed three to four hours for attachment. At that time, the filters were placed in holders, which were then put in the exposure chambers and exposed to O_3 or NO_2 at the concentration and for the times indicated below. After exposure, the filters were removed from the holders, transferred to petri dishes containing nutrient medium, and incubated for 7 to 10 days to permit colony development by survivors. Colonies were visualized by staining the filters with hematoxylin.

Measurement of Direct Effects of NO_2 on V-79 Cells

Growing cultures of V-79 cells were double-labeled with ^{14}C amino acids (0.1 µCi/ml) and ^{3}H-TdR (1.0 µCi/ml) for 24 h and then seeded onto filters, which were then placed into Lexan filter holders. After another 24 h, the cells were exposed for 2 h to NO_2

at 5 ppm. The filters were then removed from the holders, washed gently with 0.9% sodium chloride, and air dried. The radioactivity remaining with the filters was determined by scintillation counting.

EXPERIMENTAL RESULTS

Cytotoxic Effects of NO_2 and O_3

It was recognized early in these studies that V-79 cells were very sensitive to the effects of NO_2 when the gas was in direct contact with the cells (Samuelsen et al., 1978). Exposure of cells to 0.15 ppm of NO_2 inactivated their colony-forming ability in a dose-dependent manner; after 6 h, fewer than 90% of the originally exposed cells could form macroscopic colonies during subsequent incubation of the filters in immersed culture.

Ozone was somewhat more effective than NO_2 in activating colony formation by V-79 cells. Figure 5 shows the results of a series of studies with O_3 at 0.05 ppm in air. Colony-forming ability was always compared with that of cells exposed to clean air in separate chambers. In the clean-air controls, very little loss of colony-forming ability was seen (less than 10%) for exposures as long as 6 h.

Effect of NO_2 and O_3 on DNA Replication

In mammalian cells, many chemical and physical mutagens induce DNA damage that interferes with DNA replication (Painter, 1978). This can be shown by measuring, at intervals, the rate of DNA synthesis in cell cultures after a single treatment with the test agent. With most mutagens, the rate of DNA synthesis declines with time after treatment. Chemicals that inhibit DNA synthesis, but do not damage DNA, do not produce such an effect, and DNA synthesis returns to its normal rate when the inhibitors are removed.

To test for the effect of NO_2 and O_3 on DNA replication, V-79 cells were labeled with ^{14}C-TdR as described above and exposed to either O_3 (0.03 ppm, 1 h), NO_2 (0.15 ppm, 1 h), or 254-nm ultraviolet (UV) light (5 J/m^2). Immediately after treatment and one and two hours later, sample cultures from each group were labeled for 10 min with ^3H-TdR and immediately fixed with cold 5% trichloroacetic acid. The results are shown in Figure 6. The data are presented as percentages of the rates of DNA synthesis in appropriate controls (cultures exposed to clean air as controls for NO_2 and O_3 and cultures sham-exposed to UV light).

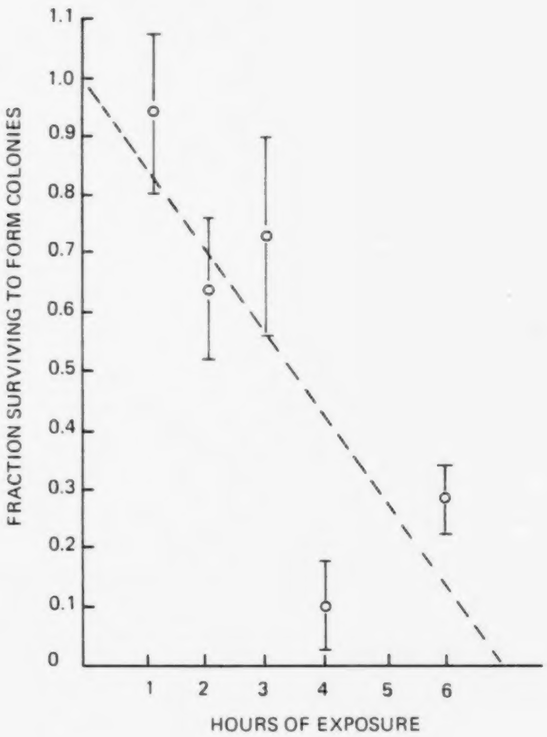

Figure 5. Inactivation of V-79 cells by 0.05 ppm ozone.

Exposure to NO_2 produced a slight decrease in the rate of DNA synthesis, but the rate returned to normal when the cultures were placed in fresh culture medium. Both O_3 and UV light also produced a slight initial decrease in the rate of DNA synthesis: but in contrast to the results with NO_2-exposed cells, the rate of DNA synthesis continued to fall during subsequent incubation in fresh medium. This suggests that O_3 may have damaged the cellular DNA.

Mechanism of Action of O_3 and NO_2 on Directly-exposed Cells

The oxidizing effects of NO_2 and O_3 on membrane lipids are well known (Goldstein and Balchum, 1967: Thomas et al., 1968): the toxicity seen in the present exposure system may be due in part to such oxidations of the cell surface membranes. Exposure of thin films of peanut oil to 0.15 ppm of NO_2 in the present system produced detectable peroxidation (Goldstein et al., 1969).

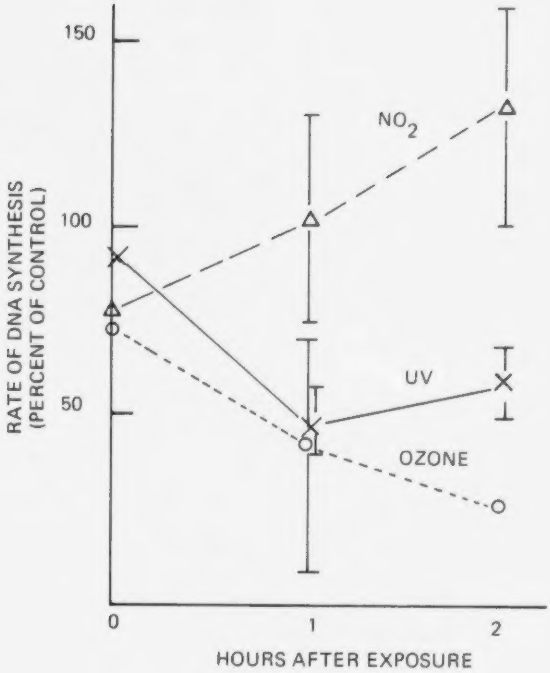

Figure 6. The effect of NO_2, ozone, and UV-light on the rate of DNA synthesis in V-79 cells.

Microscopic examination of cells after various periods of exposure to NO_2 tended to support this hypothesis. The first observable changes were that the cells seemed to become more rounded and firmly attached to the filter substrate. With longer times of exposure and higher NO_2 levels, the nuclei were distorted. Finally, 5 ppm of NO_2 produced loss of cells from the filters. A possible trivial explanation could be that NO_2 somehow affected the Millipore membrane so that it could no longer support cell growth and attachment. This was shown not to be the case through studies in which filters were exposed to 5 ppm of NO_2 for 6 h. This treatment did not alter cell attachment or the ability of the filters to support cell growth.

To obtain some quantitative information on cell destruction by NO_2, cultures of V-79 cells were labeled simultaneously with ^{14}C-amino acids and ^{3}H-TdR as described above. The doubly-labeled cells were then planted on filters so as to provide a nearly confluent cell sheet at the time of exposure to NO_2.

Table 1 gives the results of several experiments in which exposure to NO_2 at 5 ppm for 2 h produced an average loss of cells from the filters of about 50%. Both ^{14}C and ^{3}H were lost from the filters. In every case, however, proportionately more tritium was lost from the NO_2-exposed filters: the ratio of ^{3}H:^{14}C remaining on the filters was always lower for the NO_2-exposed filters. Exposure to clean air did not produce any loss of label, as shown in experiments 18, 21, and 25.

Table 1. Results of Mammalian Cell Exposure to Nitrogen Dioxide

Experiment Number	Exposure	^{3}H DPM $\times 10^{-5} \pm 1$ SD	^{14}C DPM $\times 10^{-3} \pm 1$ SD	Ratio ^{3}H:^{14}C
15	NO_2	1.72 ± 0.14	1.18 ± 0.05	14.6
	Air	7.58 ± 0.16	3.73 ± 0.10	20.3
17	NO_2	0.982 ± 0.074	3.78 ± 0.27	26.0
	Air	1.66 ± 0.19	5.88 ± 0.68	28.2
18	NO_2	0.850 ± 0.20	2.75 ± 0.57	30.9
	Air	2.50 ± 0.33	5.34 ± 0.82	46.8
	Not Exposed	2.31 ± 0.40	5.07 ± 0.91	45.6
21	NO_2	4.60 ± 0.82	25.00 ± 0.47	18.4
	Air	11.2 ± 0.79	44.50 ± 0.21	25.2
	Not Exposed	10.6 ± 0.40	44.10 ± 0.20	24.0
25	NO_2	3.62 ± 0.31	14.0 ± 1.3	25.9
	Air	5.33 ± 0.26	17.7 ± 1.3	30.1
	Not Exposed	5.55 ± 0.40	19.1 ± 1.4	29.1

DISCUSSION

This research project was begun with the goal of developing an *in vitro* analog of the respiratory epithelium. Cell culture methods were to be established for maintaining living cells in nearly direct contact with test atmospheres, while at the same time preventing the cells from drying out. In addition, these methods would allow recovery of the cells for further study. Previous systems for exposing cells to gaseous materials have relied on 1) solution of the pollutant in the medium bathing the cells or

2) periodic short exposures of the cell layer followed by immersion of the cells in liquid media.

A method for exposing cells for relatively long periods to almost any atmosphere has been developed; however, cells exposed in this system were extremely sensitive to very low levels of pollutant gases, which may not realistically indicate the effects of pollutants on experimental animals and humans. For example, O_3 and NO_2 concentrations of less than 1 ppm caused rapid destruction of cells in this test system. This great sensitivity may be related to the relatively large area of the cell surface that was exposed to the gases, since the cells were spread thinly on the membrane surface. In contrast, the cells of the respiratory tract *in vivo* are closely packed, as in the columnar epithelial areas, and only a small percentage of the total cell surface area is exposed to airflow in the airways. Also, in the normal lung the cells are covered by a mucous layer that shields them from exposure to oxidants and particulates. Simulation of this mucous layer in the present exposure system was not attempted.

The exposure system is not limited to the use of the cell cultures on membrane filters. The exposure chambers will accommodate conventional organ culture dishes, which are being used in current studies of ~~the~~ role of oxidant gases in neoplastic transformation. It is known that O_3 and NO_2 damage lung cells and induce hyperplasia (Hackett, 1979; Dungworth et al., 1975), but the role (if any) of this response in neoplasia is not clear. Using the present exposure system, we hope to provide evidence on this subject.

ACKNOWLEDGMENTS

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SESSION 2

DRINKING WATER AND AQUEOUS EFFLUENTS

PB82-233255

IS DRINKING WATER A SIGNIFICANT SOURCE OF HUMAN EXPOSURE TO CHEMICAL CARCINOGENS AND MUTAGENS?

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Drinking water has long been suspected as a medium through which chemical carcinogens and mutagens reach man. The first inquiries into this possibility were made by Heuper and Ruchhoft (1954), who tested carbon-chloroform extracts from the following samples: a gravity oil separator effluent from a petroleum refinery; raw water from a canal polluted by wastes from a petroleum refinery; raw water from Nitro, WV (the Kanawha River); and finished water from Cincinnati, OH (the Ohio River). These samples were applied topically to black male C57 mice in whole-animal carcinogenicity tests lasting one year. Samples from the refinery and the ship canal were carcinogenic; the raw water from Nitro and the finished water from Cincinnati gave negative results. While this study did not give direct evidence of carcinogens in drinking waters, it brought attention to the presence of such chemicals in surface waters used as sources for drinking water.

The development and general application of very sophisticated analytical tools in the past ten years have greatly altered our views on pollution of drinking waters. Coupled gas chromatographic and mass spectral analysis of drinking water has rapidly increased the numbers of chemicals known to occur in drinking water. Where previously the identification of a single chemical might take months or years, the general availability of mass spectrometry and the development of associated computer systems now allow almost instantaneous identification of hundreds of chemicals (if reference fragmentation patterns are available). To date, more than 1152 different chemicals have been identified in extracts of U.S. drinking waters (Melton, 1979); several of these chemicals are known to produce tumors in humans or experimental animals.

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The most significant finding in recent years is that the organic chemicals usually found at the highest concentrations in drinking waters arise not from industrial pollution, but from disinfection of drinking water with chlorine (Rook, 1974; Bellar et al., 1974). The first such chemicals identified were the trihalomethanes (THMs), primarily bromo- and chloro-substituted methanes, with occasional traces of iodomethanes. The significance of these observations was increased by the National Cancer Institute Carcinogenesis Bioassay Program's finding that chloroform is carcinogenic in rats and mice. Results from animal studies were subsequently supported by a number of studies indicating a correlation between drinking water chlorination and gastrointestinal- and urinary-tract cancer mortality (see Wilkins et al., 1979, for a review). In addition, several chemicals found in drinking water have been identified as mutagens in the Ames test (Simmons et al., 1977).

As the above findings became known, workers began to investigate the biological properties of complex mixtures of chemicals in drinking water. A number of laboratories have found organic concentrates of drinking water to be mutagenic in the Ames test (Loper et al., 1978; Glatz et al., 1978). These studies have been followed up with demonstrations that such concentrates can transform BALB/3T3 cells (Lang et al., in press). When chemicals from water are sufficiently concentrated and fractionated, it is doubtful that any surface water supply will be found to be without any mutagenic activity.

Several investigators have shown that the THMs represent only a fraction of the products of chlorination. Many substances produced by chlorination of humic and fulvic acids and isolated from water remain to be identified; many of these are not available from commercial sources. Data recently reported by Symons (in press), of the Drinking Water Research Division of the Municipal Environmental Research Laboratory, in Cincinnati, indicate that more than 50% of the organic chlorine produced through chlorination of drinking water can be in products other than THMs. Although chloramination, an alternate disinfection method, suppresses THM formation, it does not suppress the production of non-THM organic chlorine to the same extent.

Mouse skin initiation/promotion studies with various disinfectants indicate that these non-THM products cannot readily be dismissed. In one such experiment (see Table 1), tumor-initiating activity was found only for drinking water disinfected with chlorine, chloramines, or ozone (Bull, 1980). Since these concentrates were prepared by reverse osmosis, they did not contain THMs.

Table 1. Tumor Initiation by Reaction Products of Various Disinfectants[a] After 20 Weeks of Promotion with PMA[b]

Sample[c]	Concentration Factor[c]	No. Animals with Tumors	Total Tumors	% Animals with Tumors
Non-disinfected	102	0/25	0	0
Chlorine	106	4/25	5	20
Chloramine	142	5/25	8	32
Chlorine dioxide	168	0/25	0	0
Ozone	186	7/25	9	36
Saline	-	1/25	1	4
7,12-dimethyl-benzo(a)anthracene	-	16/25	35	140

[a] Substrate was settled, coagulated, and filtered Ohio River water. The total dose was 1.5 ml (6 x 0.25 ml) given subcutaneously at the concentration factor indicated.

[b] PMA = Phorbol myristate acetate applied at a dose of 2.5 µg, three times weekly.

[c] After treatment, water was subjected to reverse osmosis with cellulose acetate to the level indicated by the concentration factor (initial volume/final volume).

With this brief background, we can summarize the major categories of hazardous chemicals in drinking water:

1) Trace quantities of a wide variety of synthetic organic chemicals, usually at < 1 µg/l in surface waters.

2) Sporadic occurrence of high concentrations of individual industrial chemicals, typically involving groundwater contamination with bulk solvents.

3) Natural (or background) organic chemicals, such as humic and fulvic acids.

4) Products of the reaction of disinfectants with background chemicals (including THMs, the major class of organic compounds in drinking water).

5) Chemicals leached from distribution systems, such as lead, asbestos, and polycyclic aromatic hydrocarbons.

6) Water-treatment chemicals (including polyelectrolytes, coagulants, and corrosion-control chemicals).

The third and fourth of these items are peculiar to and ubiquitous in drinking water; they pose the greatest problems in assessing the risks associated with drinking water.

The overwhelming numbers of chemicals that appear in drinking waters, their low individual concentrations, the complications associated with drinking-water disinfection, and the need to demonstrate actual reduction in carcinogenic risk through various treatment options dictate a bioassay approach to drinking water risk assessment. The presence of several hundred to several thousand compounds in a drinking water at a fraction of a microgram per liter is the rule rather than the exception. Individually, most of these chemicals might contribute little to human disease (with the possible exception of the THMs). However, they add up to concentrations of from one to several milligrams of total organic carbon per liter of drinking water and could account for some of the hazards suggested by epidemiological data (Wilkins et al., 1979).

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PB82-233263

ALTERNATIVE STRATEGIES AND METHODS FOR CONCENTRATING CHEMICALS FROM WATER

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INTRODUCTION

The concentration of organic matter in water can range from several hundred micrograms per liter in groundwater to many milligrams per liter in industrial or sewage effluents. Generally, the biologically active materials will be present in concentrations too low to be detected by testing the small amount of aqueous sample that can be incorporated directly into a biological test system. The organic matter in drinking water and wastewater is a complex mixture and defies complete characterization by current technology. Consequently, these materials cannot be purchased or synthesized for biological testing, but must be obtained from the water to be evaluated. A paradoxical situation results, since the evaluation of methods for concentrating an organic substance depends on the existence of reliable analytical methods for the quantitative analysis of the substance. Therefore most of the data available concerning organic concentration techniques are based on performance with a few specific compounds or on general parameters such as total organic carbon (TOC) or total organic halogen.

Methods for producing samples of organics from water for biological testing can be divided into two types: concentration and isolation. In the former, water is removed, leaving the dissolved substances behind; in the latter, the organic substances are removed from the water. Combinations of methods have also been used to prepare samples for biological testing. This paper does not deal with the theoretical aspects of the methods, but rather reviews the advantages and disadvantages of representative examples of both approaches. This paper is intended as a guide to the

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interpretation of data obtained through biological testing of organic concentrates produced through methods currently in use.

The choice of approach to preparing samples for biological testing is determined by the biological test system to be used. The final sample must provide a sufficient quantity of material in a volume of solvent that is compatible with and can be accommodated by the test system. Generally, more sample is required for biological testing than for chemical analysis. Most of the methods included in this paper were developed and evaluated for preparing samples for chemical analysis and, in most cases, will have to be scaled up to prepare samples for anything other than small-scale *in vitro* tests.

CONCENTRATION TECHNIQUES

Some volume-reduction methods for preparing organic concentrates for biological testing include freeze concentration, freeze-drying, vacuum evaporation, and membrane processes (reverse osmosis and ultrafiltration). If aqueous concentrates are required, the degree to which the samples can be reduced in volume is limited by the concentration of inorganic substances in the sample and the aqueous solubility of the organic substances. If the final concentration required for testing is greater than these parameters will allow, then the volume-reduction method can be used as a first step in a combination method, as will be described later.

Freeze concentration is the process whereby a water sample is frozen into a shell of pure ice, leaving in the center the unfrozen water containing the dissolved substances. Shapiro (1961) proposed using this method for concentrating environmental water samples; it was subsequently evaluated by Baker (1969). The method allows effective recovery of all components tested, including volatile and ionized organic species. The components are equally but not totally recovered.

Freeze concentration works well in distilled water solutions, but recovery of organic solutes decreases with increasing salt content, due to alterations of the forming ice surface that result in the incorporation of solute-rich liquid into the ice (Baker, 1970). Baker demonstrated that initial concentrations of up to 310 mg/l of total dissolved solids have little effect on the recovery of the test substance m-cresol, with approximately 80% being recovered in the liquid after a 20-fold reduction in volume (Baker, 1969). Baker used a rotary evaporator with the flask immersed in a freezing bath to concentrate samples of a few hundred milliliters spiked with milligram quantities of test compounds. At one time, a freeze concentrator capable of concentrating 5-l samples up to

30-fold was available, but it is no longer manufactured. If large samples are to be freeze concentrated, the equipment will have to be custom-made and evaluated.

Freeze-drying is the process of removing water vapor directly from the frozen sample by sublimation under vacuum. Large-scale equipment is available, but it is cumbersome and expensive, and it requires large amounts of energy. Freeze-drying is a slow process. In our laboratory, we use an apparatus that allows up to 40 l of water to be processed at one time; about 72 h are required to remove all of the water. The residue remaining is composed largely of inorganic salts and is hygroscopic, so that a solvent is required to recover it from the large stainless steel pans used as sample containers.

Vacuum evaporation (or vacuum distillation) is the boiling of the aqueous sample at reduced pressure at or near ambient temperature. This method has been used to concentrate water samples for chemical analysis (Jolley et al., 1975) and for biological testing (Johnston and Herron, 1979). The required apparatus can be assembled from commercially available components.

Freeze-drying and vacuum evaporation can achieve high degrees of concentration with little contamination, and only substances volatile at the temperature and pressure used will be lost. For most environmental water samples, large percentages of TOC can be recovered. The major drawback in both cases is the difficulty of recovering the organic substances from inorganic precipitates. This problem will be addressed at the end of this section.

In reverse osmosis, water is preferentially forced through a membrane by applying an external pressure that exceeds the osmotic pressure across the membrane. Reverse osmosis systems are commercially available in many sizes and generally contain either cellulose acetate or polyamide (nylon) membranes. The polyamide membranes are more stable at extremes of pH but are highly sensitive to chlorine. They cannot, therefore, be used to process chlorinated waters unless the residual is chemically reduced. One disadvantage of the commercial systems is that they contain plastic components and synthetic adhesives that could adsorb sample components or release contaminants into the concentrate.

A schematic of a reverse osmosis system used to concentrate water samples is shown in Figure 1. The water to be concentrated is circulated past the membrane under pressure; a fraction of the volume is removed, and the remainder is recirculated to the feed tank. Cellulose acetate membranes commonly used are rated to reject organic substances of molecular weight greater than 200 and 90 to 97% of the inorganic ions. Generally, this degree of inorganic rejection is achieved. However, while molecular weight

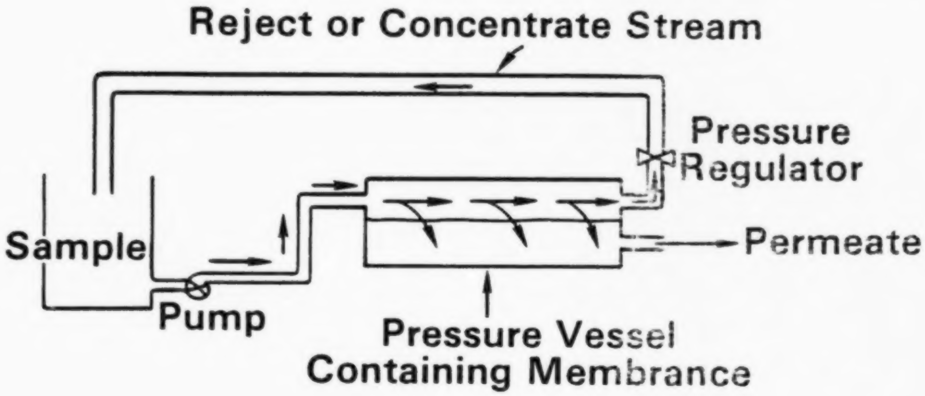
Reject or Concentrate Stream
Pressure Regulator
Sample
Permeate
Pump
Pressure Vessel Containing Membrance

Figure 1. Schematic of reverse osmosis concentrator.

influences the rejection of organics by the membrane, polar or ionized species are rejected more effectively than hydrophobic nonionized substances. In contrast to the other volume-reduction methods described, reverse osmotic concentration works through an exponential decay process in the recirculating system. Figure 2 illustrates the percent recoveries obtained for compounds with rejections of 70, 80, and 90% at volume reductions between 10- and 1000-fold. Table 1 shows the actual concentration factors obtained when sample volume is reduced 10-, 100-, and 1000-fold. While reverse osmosis does not retain all of the substances equally, it does retain much of the organic carbon in the drinking water samples.

Each of these concentration methods has its own advantages and disadvantages, but the one disadvantage shared by all of these methods is that inorganic species are concentrated along with the organic substances of interest. The degree to which samples can be concentrated by these methods before precipitation of inorganics occurs varies with the types and concentrations of inorganics originally present, but is generally 20- to 50-fold. If the bioassay system is sensitive enough and will tolerate aqueous samples, and the inorganic salts do not interfere, these concentrates can be tested directly. Freeze concentration and reverse osmosis produce only such aqueous concentrates; if more concentrated samples are required, the samples must be processed

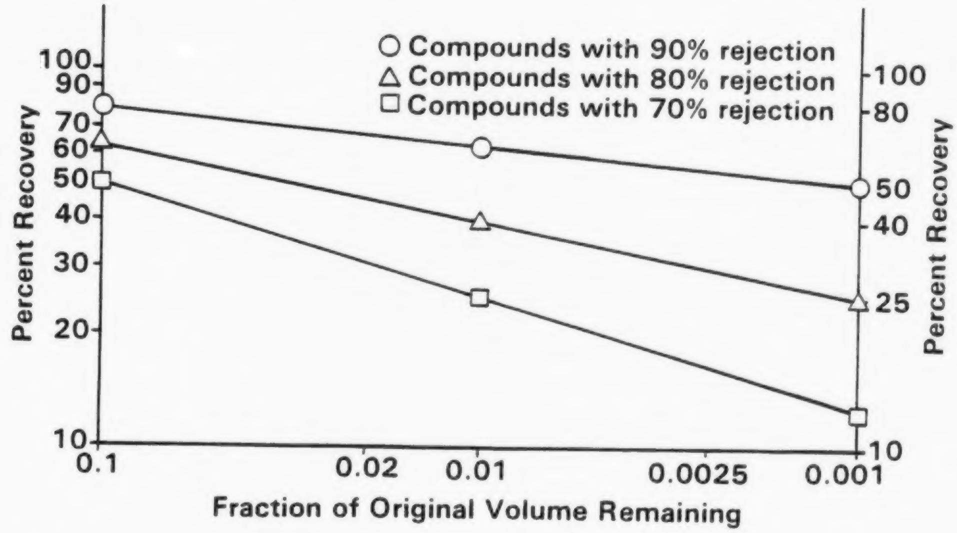

Figure 2. Recovery of compounds by reverse osmosis at various stages of concentration.

Table 1. Effect of Percent Rejection on the Actual Concentration of Constituents Obtained by Reverse Osmosis

	Actual Concentration Factor		
	Fraction of Original Volume Remaining		
Compound Percent Rejection	0.1	0.01	0.001
100	10	100	1000
90	8	60	500
80	5.2	40	250
70	5	25	125

further by other means. Freeze-drying and vacuum evaporation can be used to produce either aqueous concentrates or a solid residue. If inorganic constituents precipitate, recovery of the organics from the residue is difficult.

Pitt and Scott (1973) concentrated effluent from sewage-treatment plants by vacuum evaporation followed by freeze-drying. Many of the salts were carbonates and could be solubilized in acetic acid; samples were concentrated 2000-fold, with more than 95% recovery of TOC. River and lake waters were concentrated by the same methods. Because the starting levels of organic carbon were much lower than in sewage, a concentration factor of 10^4 was required. The inorganics were not predominantly carbonates and could not be redissolved; consequently, about 75% of the organic carbon originally present could not be recovered. Extraction with methanol gave only slightly better yields. Recovery studies were conducted with 13 compounds representing the classes of compounds expected in natural water samples. Table 2 shows the percentages of these compounds recovered from a spiked river water sample. These workers improved the recovery of organic carbon by passing the water sample through a weak cation exchange column prior to the evaporative steps. Losses of organic carbon from four samples thus treated still ranged from < 10 to 57%.

Crathorne et al. (1979) studied the recovery of 5-chlorouracil, 5-chlorouridine, 4-chlororesorcinol, and 5-chlorosalicylic acid from residues obtained from spiked samples of finished drinking water. The 14 solvents listed in Table 3 were investigated for efficiency in recovering the compounds from the residue. Only water and methanol yielded greater than 90% recovery, but the mean recovery with methanol was about 60% (B. Crathorne, Water Research Centre, Medmenham Laboratory, Manlaw, Buckinghamshire, England, personal communication, 1980).

Volume-reduction methods are most successful if precipitation is prevented during concentration. This has been accomplished by Pitt as described above, by Kopfler et al. (1977), using Donnan dialysis to exchange sodium ions for calcium ions during reverse osmosis, and by Johnston and Herron (1979), through a method to be described below.

ISOLATION TECHNIQUES

Isolation methods remove organics from water by concentrating them in organic solvents. One method is to use immiscible organic solvents to extract the water; another is to adsorb the organics onto a solid medium and elute them with an organic solvent. Many variations of these methods have been investigated, because they have been used extensively to prepare samples for chemical analysis.

Table 2. Recovery of Individual Compounds in Aqueous Solution after Evaporation-Lyophilization[a]

Compound	Anticipated Final Concentration (μg/ml)	Percent Recovered
Sucrose	10	>50[b]
Uracil	10	80
Guanosine	10	5
Xanthine	8	10
Uric Acid	25	<5
Hippuric acid	20	5
p-Cresol	13	0
p-Hydroxyphenylacetic acid	15	5
Syringic acid	15	15
p-Hydroxybenzoic acid	18	10
o-Chlorobenzoic acid	40	20
p-Chlorobenzoic acid	40	0
o-Chlorophenol	33	0

[a] From Pitt and Scott (1973).

[b] Quantification uncertain due to interference of naturally occurring compound.

Table 3. Solvents Tested for Extracting Concentration Residues[a]

Solvent	Concentration Residue
Methanol	Diethyl ether
Water	Dimethylsulfoxide
Acetone	Dioxane
Acetonitrile	Ethyl acetate
Carbon tetrachloride	Isopropanol
Chloroform	Pyridine
Dichloromethane	Tetrahydrofuran

[a] B. Crathorne, Water Research Center, Medmenham Laboratory, Manlaw, Buckinghamshire, England, personal communication, 1980.

Direct liquid-liquid extraction is suitable for the recovery of organics from water samples of several liters. Large samples, however, require continuous extractions using large volumes of solvent or refluxing a smaller volume of solvent to provide pure extractant.

As with any process employing solvents, impurities can be concentrated along with sample components. Most highly purified organic solvents contain preservatives--often an antioxidant--that can react to add organic contaminants to samples. For example, cyclohexene is an impurity present in the best grades of methylene chloride. When methylene chloride is used to extract samples that contain a chlorine residual (as do most drinking waters and wastewaters), the cyclohexene produces mono-, tri-, and tetrachlorocyclohexenes and cyclohexanes by reacting with the chlorine residual (Logsdon et al., 1977). Also, peroxides may contaminate extracts prepared with ether or may react with sample components to produce new substances not originally present in the sample or solvent. Another critical area for investigation is whether changes in the organic residues could occur in concentrates of organics during storage between preparation and analysis or biological testing.

The adsorption-elution methods require the least-complex apparatus for isolating organics from water. The water sample is passed through a column of the solid adsorbant, and the organics are subsequently eluted with a smaller volume of suitable solvent. The most common adsorption-elution methods used are activated carbon, ion exchange resins, macroreticular resins, and reverse phase high-performance liquid chromatography columns.

Activated carbon has been used to remove organics from aqueous solutions (Buelow et al., 1973). However, recovery from carbon is not as good as can be obtained with other media (Chriswell et al., 1977). Formerly, organics were recovered from the carbon through air-drying followed by prolonged Soxhlet extraction with chloroform and ethanol. This method has been abandoned because of the air-drying step and the continued boiling of the extract in the extraction apparatus. A procedure using supercritical liquid carbon dioxide as a solvent has recently been developed and is being evaluated as a means of regenerating granular activated carbon used in water treatment (Modell et al., 1978). The liquid carbon dioxide is miscible with water, allowing the drying step to be eliminated. The extraction takes place at about 30°C in a closed system, eliminating the high temperature encountered with organic solvents. The liquid carbon dioxide has also been shown to be a good solvent for several classes of organics. This method has not yet been evaluated for producing extracts for bioassay.

The XAD resins produced by Rhom and Haas have been used extensively to recover organic substances from water. Two types are available: a styrene-divinyl benzene copolymer and a methacrylate-based copolymer (Dressler, 1979; Gustafson and Paleos, 1971). Resins of both types are available in various pore sizes, giving different unit surface areas. These resins are produced for industrial use and contain many lower-molecular-weight contaminants. The resins must be prepared for laboratory use by serial extraction in a Soxhlet extractor with methanol, diethyl ether, and acetonitrile (Junk et al., 1974). Before use, the resins should be evaluated to insure that the extraction has, in fact, removed contaminants from the resin to the degree required. If resin beads are allowed to dry out, they can crack, exposing newly contaminated surfaces; thus, clean resin should be stored under methanol until used.

XAD resins have been used to isolate synthetic organic chemical contaminants from water for chemical analysis. They have a great affinity for hydrophobic substances and retain virtually all of these materials, even when the aqueous sample is passed through the resin column at high flow rates (Junk et al., 1974). Much of the organic matter in water is hydrophilic, however; Thurman and his co-workers (1978) have demonstrated that the capacity of the resins for these compounds is not great and that flow rates during the adsorption step must be in the range of 15 to 20 bed volumes/h for good recovery. Organic acids and bases are effectively adsorbed from water only after ionization has been suppressed by pH adjustment. Lowering the pH to protonate the organic acids generally presents no problem, but attempting to recover organic bases at a high pH can result in clogging of the column by inorganic hydroxides after only a small amount of water has passed through the column. It has been estimated that about 50% of the TOC in the average water sample can be concentrated onto a column of XAD-8 resin (Malcolm et al., 1977).

Ionizable organic substances can be recovered by elution with aqueous solutions of inorganic acids or bases. This will give an aqueous solution more concentrated than the original sample, but further concentration may still be required for biological testing. Elution with organic solvents is effective for recovering neutral organic substances. A variety of solvents have been used to elute the adsorbed organics from XAD columns; the most commonly used are diethyl ether, methanol, acetone, methylene chloride, and mixtures of these solvents. As discussed for liquid-liquid extraction, precautions must be taken to prevent impurities in the organic solvents from producing artifacts in the sample.

Workers from the U.S. Geological Survey have also found that humic and fulvic acids are adsorbed onto both types of XAD resins at pH 2 (Aikin et al., 1979). However, about 20% of the material binds irreversibly to XAD-2 resin and cannot be eluted with alkali or organic solvents. Much of the organic matter in surface waters is composed of these naturally occurring acids or derivatives produced during water disinfection. Because they can bind many lower-molecular-weight organic substances, these acids should be recovered to insure recovery of these bound materials. Owing to its simplicity, it is tempting to use the resin technique for producing samples for biological testing; however, it must be remembered that not all of the organic substances are adsorbed and those that are may not be fully recovered.

COMBINATION METHODS

Methods for isolating organics from water for biological testing include combinations of several techniques, for convenience or in attempts to approach 100% recovery of organics from the water.

Kopfler et al. (1977) use reverse osmosis to reduce the volume of water samples from thousands of liters to about forty liters. To prevent precipitation of inorganic salts, sodium ions are exchanged for calcium and magnesium ions in the concentrate through a Nafion tubular membrane (Dupont) concurrently with the reverse osmosis process. The concentrated aqueous sample is then transported to the laboratory, where it is extracted with pentane and methylene chloride and passed through a column of XAD-2 resin, which is eluted with ethanol. Recovery of organics by this method is estimated to be 35 to 40%. Residues have been tested for mutagenicity and cellular transformation *in vitro* and teratogenicity and carcinogenicity *in vivo*.

Johnston and Herron (1979) first pass the water through a "parfait" column containing layers of silica gel, cation exchange resin, and anion exchange resin. This step results in the adsorption of some neutral hydrophobic organics as well as ionized species including inorganic ions. The effluent from the column is evaporated under vacuum to concentrate the hydrophilic nonvolatile organic substances. Substances adsorbed on the column are recovered by separating the layers in the column and eluting each with 2 *M* triethylammonium carbonate buffer followed by acetone. Samples prepared by this method have been tested for mutagenicity *in vitro*.

Baird and co-workers (1980) use a series of stainless-steel columns packed with microparticulate-sized weak ion exchange resins and XAD resins. They report 85 to 90% removal of TOC from highly treated wastewater passed through this series of columns. The columns are eluted with acetonitrile and a 4.5-*M* sodium chloride solution containing acetonitrile. Since the presence of acetonitrile interferes with the TOC analysis, the actual recovery of organics cannot be determined. The saline eluates of the columns are concentrated further by extraction with acetonitrile at pH 7 and again at pH 1. After these extractions, the saline solution is still colored, indicating that organics have not been completely recovered. These extracts have been tested for mutagenicity *in vitro*.

Probably the most elaborate device for recovering organics from water for toxicity testing is that developed in France by Carbridenc and Sidka (1979). The apparatus is designed to extract 1000 liters of water with 100 liters of chloroform under an inert gas. The water is extracted first at pH 7, then at pH 2, and finally at pH 10. The aqueous sample is then neutralized and passed through small columns containing anion exchange resin (eluted with butanol), XAD-2 (eluted with a mixture of ethanol and methylene chloride), and activated carbon (washed with ethanol and extracted with chloroform). The recovery of 51 substances was determined using a 100-l version of this apparatus. Forty-five of the compounds were detected in one or more of the fractions, and total recovery was calculated to be 88% by weight. Extracts of drinking water prepared by this method have been tested for cytotoxicity *in vitro* and for promotion *in vivo*.

CONCLUSIONS

The results from biological tests of organic concentrates in water can be used to estimate the hazards associated with the water, but only to the degree that the concentrate represents the organic materials actually present in the water. The concentrate should contain representative amounts of all the organic materials originally present, or at least a predetermined fraction of them. Also, the integrity of the chemicals must be maintained, with no contaminants present, or at least none that interfere with the biological tests. Until such methods or combinations are developed and validated, the information in this paper should serve as a guide to the representativeness of concentrates produced by various methods and should allow proper reservations to be made in the interpretation of biological test results.

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DETECTION OF ORGANIC MUTAGENS IN WATER RESIDUES

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INTRODUCTION

In previous studies (Loper and Lang, 1978; Loper et al., 1978; Lang et al., in press; Kurzepa et al., in press), we used short-term bioassays to demonstrate the mutagenicity, carcinogenicity, and toxicity of residues prepared from samples of drinking water from six U. S. cities. The samples were processed by Gulf South Research Institute (New Orleans, LA), using reverse osmosis plus XAD resin sorption-desorption as described by Kopfler et al. (1977). Using the Ames test, we found city-specific patterns of dose-dependent mutagenesis that were essentially independent of the microsomal activation system. One or more samples from each city showed reproducible transformation frequencies at least three times the spontaneous frequency. Focus formation induced by these samples was equivalent to malignant transformation as verified in nude mice. In these studies, quantitation of mutagenic and transformation responses were complicated by the toxicity and heterogeneity of the complex residue mixtures.

These findings justify further efforts at compound identification, and for that purpose, we have proposed the development of a coupled bioassay/chemical fractionation procedure (Loper and Lang, 1978). Such a method would be patterned after successful analyses of other complex environmental mixtures such as synthetic fuels (Guerin et al., 1978). Mutagenicity would be assayed using the Ames test. Initial partitioning of the sample by liquid/liquid extraction would be followed by high-performance liquid chromatography (HPLC) for separation into smaller subfractions. Active fractions sufficiently free of inactive and

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biocidal components would be analyzed by gas chromatography/mass spectrometry (GC/MS) for identification of peaks.

Drinking water residues are not usually generated in sufficient quantity for developing such methods. However, from Mr. Francis Middleton we obtained residue still on hand from previous use of the U. S. Public Health Service carbon-chloroform extractor. This mega sampler was used in the 1950's to early 1960's for the processing of 100,000-gal samples of drinking water and source water. The sample is a liquid solution in chloroform ($CHCl_3$) of 125 g of residue obtained about 1960 from 50,000 gal of drinking water (Middleton et al., 1962). When the $CHCl_3$ is removed under a stream of nitrogen, the residue has the sticky consistency of water residues recovered from XAD resins. We have termed this material the "carbon-chloroform extracted organics" (CCEO). In precise composition, it may or may not closely resemble residues from drinking water today, but for our work, it is invaluable as an abundant supply of a complex mixture of water residuals.

Using this CCEO, we have attempted to develop a general method of coupled bioassay/chemical fractionation for separating mixtures from current drinking water. With such a method we could test immediately whether the mutagenicity of such mixtures in the Ames test is due to summation of the low activity of many mutagens or to the effects of a few relatively active components. The method should allow isolation of active subfractions in yields suitable for compound identification. Some significant biohazardous properties of residue components may not be detected by our bioassay procedure of bacterial mutagenesis plating. For example, co-carcinogens, carcinogen promoters, and certain procarcinogens and teratological agents would not be recognized. So that other subfractions could be tested using short-term *in vitro* and *in vivo* mammalian assay systems, the method should permit maximum recovery of the total sample, distributed into multiple subfractions.

METHODS AND RESULTS

Preliminary Characterization and Primary Partitioning of CCEO

Aliquots of CCEO were shown to be reproducibly stripped of all toxic traces of $CHCl_3$ by streaming with dry nitrogen for 60 min at 60°C. Mutagenesis testing of the residue using *Salmonella* strains TA98 and TA100, as described elsewhere (Loper et al., 1978), revealed dose-related, microsomal-activation-dependent TA100 mutagenesis, with some toxicity at higher doses. Following experimentation with aqueous extraction of the residue from the original $CHCl_3$ solutions and from solutions in methylene chloride (CH_2Cl_2) (Tabor and Loper, 1980), a procedure was adopted for the semisolid/liquid extraction of neutrals, acids, and bases into hexane. The resulting distributions of weight and mutagenic activity for TA98 and TA100 with and without microsomal activation have been detailed elsewhere (Tabor et al., 1980). The neutrals are one third of the total sample by weight but contain nearly all of the mutagenicity for TA100. All subsequent procedures were conducted on portions of this CCEO neutral sample.

Method Development

Quantities of partitioned samples taken for mutagen isolation had to be sufficient for repeated cycles of separation and bioassay. To obtain useful data on both mutagenicity and toxicity with minimum loss of material, tests were limited to single plates of four dose levels chosen to induce colony counts of two to three times those seen spontaneously (Loper, 1980). Reverse-phase HPLC, employing mixtures and gradients of water/acetonitrile, was used for chemical separation. The instrument was a Waters Associates ALC/GPC 204 equipped with two 6000A pumps, UK6 injector, solvent programmer, and 254-nm absorbance detector. A guard column (3.9 mm x 2.5 cm) packed with pellicular particles bonded with octadecylsilane (BONDAPAK--C_{18}/CORASIL; Waters Associates) was followed by a radial compression module (RCM; Waters Associates) containing a 8-mm x 10-cm column packed with 10-μm silica particles bonded with a high load of octadecylsilane. To achieve adequate separation levels, various gradient elution conditions were investigated using the analytical column, as sample quantities were increased from microgram to milligram levels. One milligram of the CCEO neutral sample induced approximately 2000 TA100 colonies in our assay, and for RCM chromatography, 20-mg samples were routinely loaded in 200-μl volumes of acetonitrile.

A flow diagram of our procedure for partitioned samples is given in Figure 1. Activity losses accompanying removal of the bactericidal acetonitrile were minimized by solvent exchange into CH_2Cl_2 using SEP-PAKS (Waters Associates) packed with μBONDAPAK-C_{18} (Waters Associates) followed by evaporation of the CH_2Cl_2 at 40°C under dry nitrogen in the presence of a small volume of dimethylsulfoxide. Replicate separations reproduced the HPLC fingerprint at the top of Figure 2, and with this added material, we assayed fractions 1 through 6 using both TA98 and TA100 with and without microsomal activation. As before, all the activity was detected with TA100 and appeared in fraction 5B. Rechromatography of this region gave the isolated major peaks at the bottom of Figure 2. Subfractions 5B/5 and 5B/6 contained all the mutagenic activity, and each of these was rechromatographed (Figure 3). Such subfractions are currently undergoing further study; some preliminary data are presented here. In one series of four dose-level determinations, the summed mutagenesis from the active subfractions (5B/5/2, 5B/6/2, 5B/6/3) was approximately half that

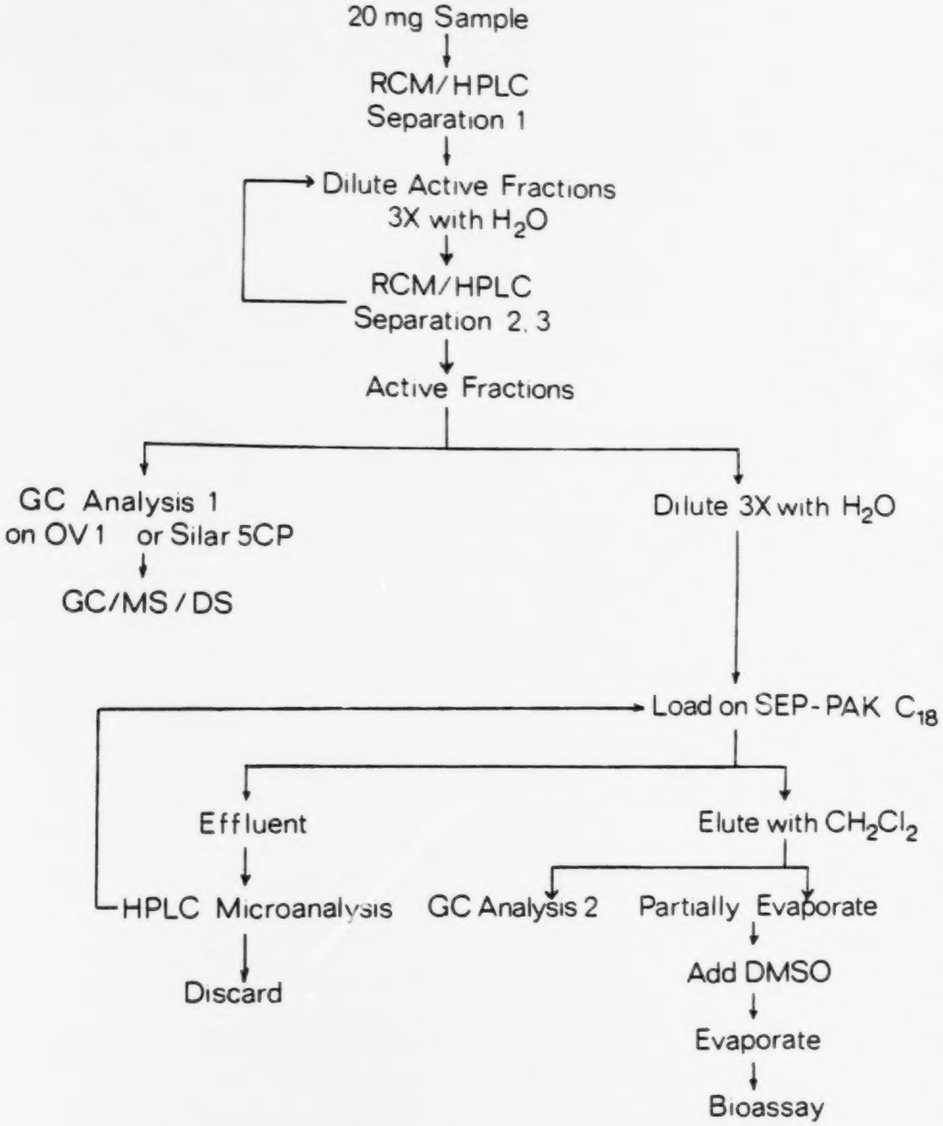
20 mg Sample
RCM/HPLC Separation 1
Dilute Active Fractions 3X with H_2O
RCM/HPLC Separation 2, 3
Active Fractions
GC Analysis 1 on OV 1 or Silar 5CP
GC/MS/DS
Dilute 3X with H_2O
Load on SEP-PAK C_{18}
Effluent
Elute with CH_2Cl_2
HPLC Microanalysis
GC Analysis 2
Partially Evaporate
Discard
Add DMSO
Evaporate
Bioassay

Figure 1. Flow diagram for coupled bioassay/chemical fractionation of partitioned complex mixtures.

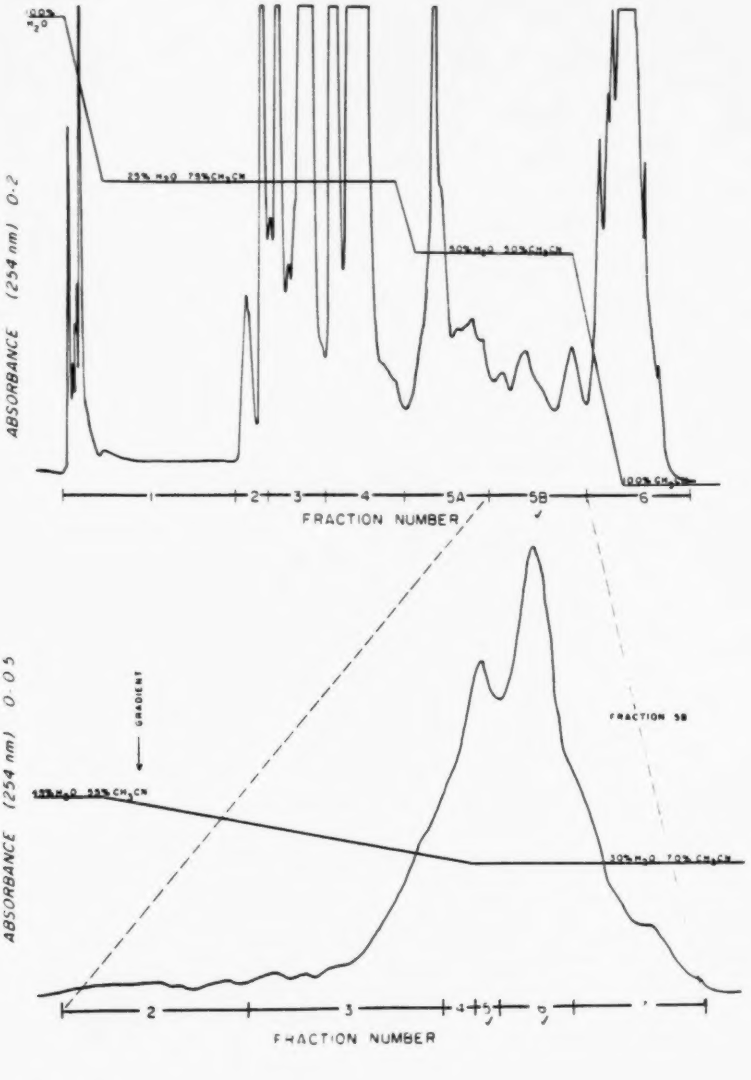

Figure 2. RCM/HPLC reverse-phase separation of CCEO neutrals. A 20-mg sample was fractionated, using gradient elutions as shown, and rechromatographed by the procedures given in Figure 1. Subfractions mutagenic to TA100 in the presence of microsomal activation are indicated by a check (✓).

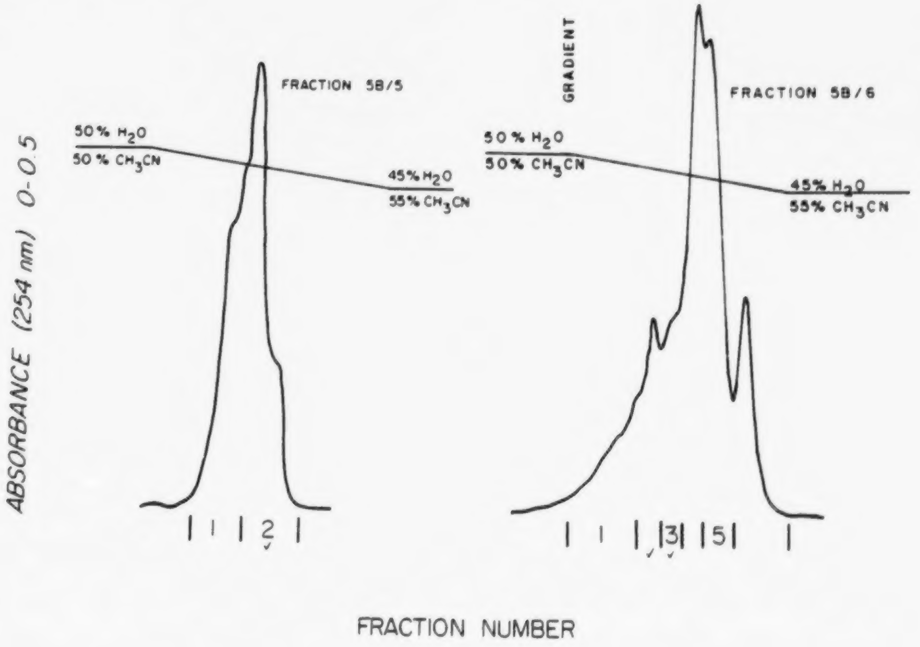

Figure 3. Fractions 5B/5 and 5B/6, generated as shown in Figure 2, were rechromatographed separately. Mutagenic subfractions are indicated by a check (✓).

from the initial 20-mg sample. The relative purity of residue in each subfraction was estimated by GC analysis. Tracings of GC chromatograms obtained using a column containing 10% SE30 on Chromosorb WHP 80/100 mesh (Applied Science) as a stationary phase are shown in Figure 4.

Subfractions 5B/5/2 and 5B/6/2 each contained two different components, and one other component constituted subfraction 5B/6/3. To date, three 20-mg aliquots of CCEO neutrals have been carried through the procedure as far as peak separation and GC analysis, using both polar (10% Silar 5CP on Chromosorb Q 80/100 mesh: Applied Science) and nonpolar stationary phases (10% SE30 on Chromosorb WHP 80/100 mesh or 3% OV1 on Chromosorb W 80/100 mesh: Applied Science): the patterns shown in Figures 3 and 4 are representative of the purity of all three aliquots.

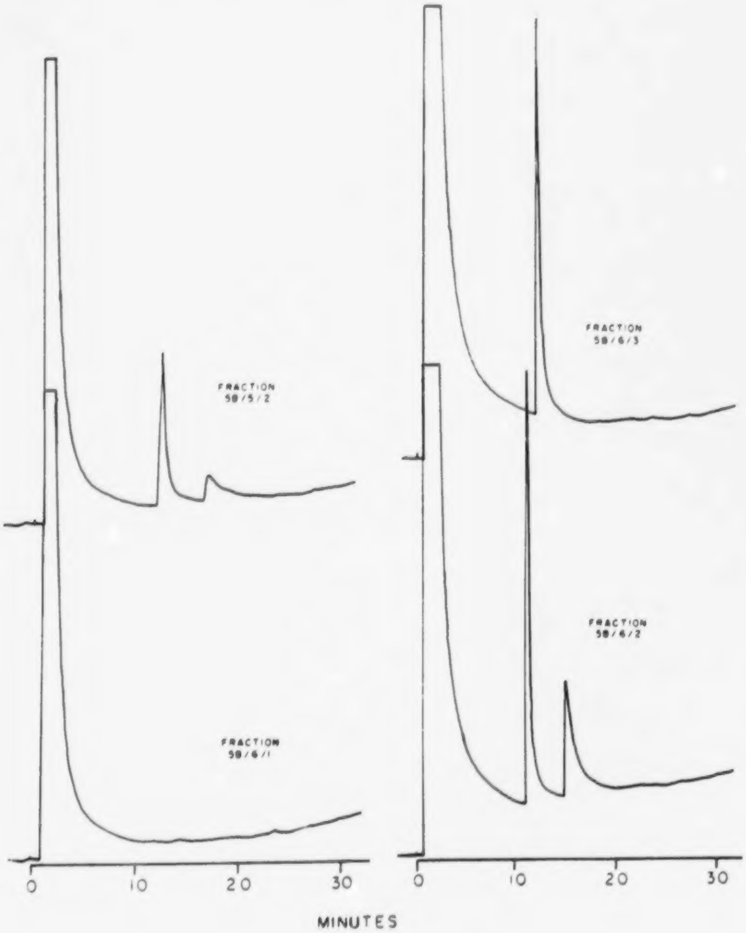

Figure 4. Flame-ionization gas chromatograms of RCM/HPLC subfractions. Seven and one-half microliters of a water/acetonitrile solution (about 50:50 by volume) of each subfraction was slowly injected into a Perkin-Elmer Model 900 GC fitted with a 2-mm x 1-m stainless steel column containing 10% SE30 on Chromosorb WHP 80/100 mesh. The nitrogen carrier gas flow was at 18 ml/min, and the temperatures of injector and detector were 280°C and 350°C respectively. A linear temperature program (120°C to 220°C at 4°/min) was initiated at the time of injection. Data were collected continuously and analyzed using a Spectra Physics Autolab System I Computing Integrator, and chromatograms were displayed on a 10-mV recorder at an attenuation of 160.

Weight values for a sample constituents were calculated based on peak areas compared with peak areas obtained from chromatography of 1 μl of a 1 mg/ml solution in chloroform of American Oil Chemists Society Reference Mixture No. 6, run under conditions identical to those for the experimental samples. These weights have been used in estimating specific activity, in net revertant colonies per milligram, shown in Table 1 (see Figure 4). Thus for 5B/5/2 and for 5B/6/2, the weights of their two GC components have been combined, so that results are expressed as net revertant colonies per total weight of each subfraction. Preliminary GC/MS analyses indicate that some of these compounds are polyhalogenated: this is indicated in Table 1 by an X with the number of components for each subfraction.

Table 1. Response of TA100 to Mutagens in the CCEO Neutrals Mixture

	Net Revertant Colonies (+S-9)	
RCM/HPLC Subfractions[a]	Total	Per mg
5B/5/2 (1X + 1)	6000	3 X 10^5
5B/6/1 (?)	?	--
5B/6/2 (1X + 1)	4600	10^5
5B/6/3 (1X)	13000	4 X 10^5

[a] Number of major peaks in parentheses: X indicates the presence of halogen(s), based on preliminary MS data.

DISCUSSION AND CONCLUSIONS

We have demonstrated that this coupled bioassay/chemical fractionation procedure is reproducible for this complex residue mixture, and we feel it will serve as a general method. For the present, we assume that concentration methods yield residues representative of the non-volatile organics in drinking water. Based on our observations in the six-city study and our results from CCEO neutral sample, drinking water residues appear to contain a vast number of non-mutagenic compounds, possibly some of low mutagenicity, and a few highly mutagenic compounds. Evidence for this is summarized in Table 2. Of the RCM/HPLC subfractions of the CCEO neutrals, only three were mutagenic. Peak 5B/6/3, containing a single component by GC, showed a specific activity comparable to those of the carcinogens β-naphthylamine or 3-methylcholanthrene (McCann et al., 1975). The other two subfractions contained two GC components each, and their mutagens must have been comparably potent (see Table 1). Of course, these subfractions were derived from the old CCEO sample. For more recently isolated residues, the presence of highly active compounds was suggested by our data for the XAD eluate of Seattle sample 1. This fraction showed direct-acting mutagenesis, which was increased 24-fold in specific activity (revertant colonies per milligram of sample material) by extraction into hexane (Loper et al., 1978: see Table 2). The specific activity of the active compound(s) might be considerably higher, based on our observation that this hexane-extracted subfraction yielded 20 major 254-nm absorption peaks through HPLC (Tabor et al., 1980).

Table 2. Mutagenesis of Drinking Water Residue Fractions and Known Carcinogens in the Ames Test (TA100)[a]

Test Substance	Net Revertant Colonies/Plate/mg
XAD eluate of Seattle drinking water (sample 1)	4×10^2
Hexane extract of XAD eluate of Seattle drinking water (sample 1)	10^4
CCEO neutrals fraction 5B/6/3	4×10^5
β-propiolactone	5×10^4
β-naphthylamine	6×10^4
3-methylcholanthrene	2×10^5

[a] Data for XAD eluate and subfraction is from Loper et al., 1978: that for known compounds is from McCann et al., 1975.

Final identification of the mutagenic components in the CCEO may be of some direct benefit, even though the sample is now nearly 20 years old, and some of the halogen content may be due to storage in $CHCl_3$. Identification by GC/MS is in progress. Should previously unidentified mutagens be detected, their characterization by MS would permit data searches, by peak recognition, among MS profiles of residues of current drinking water samples.

We propose to apply our procedure of coupled bioassay/chemical fractionation to the identification of mutagens in recently derived drinking water residues, obtained by desorption from XAD. Should major mutagens in these samples be identified, a number of questions concerning water quality could be investigated. These topics include the reduction or avoidance of mutagens by alternate disinfection procedures; seasonal changes in the types and amount of mutagenic activity; and the mutagenic potential of discharges of diverse industrial processes into surface or ground water destined for human use. Knowledge of specific mutagens in a water sample would provide an important criterion for evaluating alternate residue-isolation procedures and could lead to simplification of analytical chemical detection methods. Identification of significant non-volatile bacterial mutagens in water would be a step toward toxicological assessment of their risk to man.

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SHORT-TERM METHODS FOR ASSESSING *IN VIVO* CARCINOGENIC ACTIVITY OF COMPLEX MIXTURES

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INTRODUCTION

The carcinogenic activity of a chemical or a complex mixture derived from an environmental sample is assessed most efficiently through a three-tier decision scheme (Bridges, 1973; Weisburger and Williams, 1977; Bull and Pereira, in press). In tier 1, the samples are screened for evidence of carcinogenic and mutagenic activity. The Ames Salmonella mutation bioassay and in vitro and in vivo cytogenetic assays appear to sucessfully identify most chemicals and samples with carcinogenic activity. These two types of assays detect the two major classes of genotoxic agents, mutagens and clastogens, and would therefore form the backbone of tier 1. Other possible assays for tier 1 include mammalian cell mutation, sister-chromatid exchange, micronuclei, and unscheduled DNA synthesis. The nature of tier 1 bioassays--especially their lack of correlation to carcinogenic potency, the absence of a direct demonstration of cancer or neoplasia, and the number of false positives--requires that carcinogenic activity be confirmed in tier 2.

Tier 2 is the level in the decision tree where false positives are eliminated, insuring that the time-consuming and expensive tier 3 bioassay is used only for chemical and environmental samples that are virtually certain to contain carcinogenic activity. The function of the tier 3 bioassay is to provide data for assessing the quantitative risk associated with the carcinogenic activity of chemicals and samples. To be effective, tier 2 must confirm carcinogenic activity with a minimum of false positives, while maintaining an acceptably low level of false negatives.

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Quantitation of carcinogenic activity in environmental samples of complex mixtures, and thus ranking of mixtures according to carcinogenic activity, poses a unique problem. Lifetime feeding or exposure studies with rodents in a bioassay following the National Cancer Institute (NCI) protocol is presently the only acceptable procedure for obtaining data on carcinogenic potency that may be extrapolated to humans (IRLG, 1979). This type of bioassay is not feasible with most complex mixtures derived from drinking water and other environmental samples. For example, the expense of obtaining a single drinking-water sample for an NCI bioassay greatly exceeds that of the bioassay itself, potentially costing millions of dollars. Furthermore, the uniqueness of each drinking-water sample (containing thousands of unknown chemicals) prevents the generalization of the bioassay results to other drinking waters (Bull et al., in press). The composition of drinking waters varies depending on treatment (especially disinfectant), source (ground or surface), seasons, and the effects of industrial use and municipal waste disposal. Therefore, each drinking water would have to be considered a unique test substance, and each would require a separate bioassay, costing millions of dollars, at least until enough assays were performed to determine whether generalizations among drinking waters were possible.

CARCINOGENESIS TESTING MATRIX

The lack of a tier 3 bioassay for complex mixtures derived from environmental samples means that any ranking or quantitation of carcinogenic activity must be obtained at tier 2. To accomplish this, tier 2 bioassay results would have to relate quantitatively to carcinogenic potency. Also, since no short-term bioassay appears to be sensitive to all chemical carcinogens, a Carcinogenesis Testing Matrix (CTM)(Bull and Pereira, in press) has been proposed for tier 2. It includes mouse lung adenoma (Shimkin and Stoner, 1975; Stoner and Shimkin, in press), mouse skin initiation/promotion (Slaga et al., in press), rat liver foci (Pereira, in press), *in vitro* cell transformation (DiPaolo, 1979; Styles, 1979), and *in vivo* sister-chromatid exchange (Latt et al., 1979). These bioassays were chosen because evidence, or some reasonable rationale, indicates that their results will relate to carcinogenic potency, at least when taken together. The *in vivo* bioassays appear most attractive, because their results should be influenced by the same pharmacokinetic and metabolic factors as in lifetime-exposure carcinogenicity bioassays. Where possible, bioassays that directly measure the acquisition of neoplastic properties, such as benign tumors, preneoplastic lesions, and cell transformation, are included in the CTM.

The increased sensitivity of the CTM bioassays (compared with lifetime exposure bioassays) greatly reduces the sample requirement.

These bioassays require a few applications of sample, at most. This increased sensitivity can be illustrated with the rat liver foci bioassay (Pereira, in press). Briefly, the bioassay protocol is as follows:

1) the sample is administered by any convenient route in single or multiple doses;

2) the rats are given 500 ppm sodium phenobarbital in their drinking water, starting four to seven days after the sample and continuing for seven weeks; and

3) the rats are then sacrificed, and their livers excised and examined histochemically for foci of gamma glutamyl transpeptidase.

A two-thirds hepatectomy can be performed either 18 to 24 h before or 14 days after administering the sample. Performing the partial hepatectomy 18 to 24 h before exposure increases the spectrum of chemical classes of carcinogens to which the assay is sensitive and increases the response. Performing it 14 days after exposure also increases the response and keeps the initiation step distinctly separated from the promotion.

Table 1 shows the results for diethylnitrosamine (DENA) in the rat liver foci bioassay. A single dose of DENA (30 mg/kg) gave positive results when administered either 24 h after or 14 days prior to partial hepatectomy. When the partial hepatectomy was performed 14 days after DENA exposure, a single dose as low as 300 µg/kg was detected. Since partial hepatectomy 24 h prior to the DENA increases sensitivity to DENA (Scherer and Emmelot, 1976), one would expect an even lower single dose to be detected. Ten low daily doses of 300 µg/kg, for a total dose of 3 mg/kg, appear to be additive in the bioassay (Table 1). The additivity of multiple low doses can also result in increased sensitivity (Ford and Pereira, in press). The use of tumor promoters, preneoplastic lesions, and benign tumors in short-term bioassays greatly decreases the amount of sample required so that acquiring sufficient amounts of sample becomes feasible.

DIFFICULTIES IN RANKING POTENCY FROM SINGLE BIOASSAYS

The use of two or more bioassys to rank the carcinogenic activities of complex mixtures could result in different rankings in the different bioassays, or in some mixtures giving a positive result in one bioassay while other mixtures were positive only in another bioassay. Table 2 outlines a hypothetical situation where four environmental samples of complex mixtures (1, 2, 3, and 4) were tested in bioassays A and B. The possible results include

Table 1. Rat Liver Foci Bioassay of Diethylnitrosamine (DENA)

DENA[a] (mg/kg)	No. of Animals	Partial Hepatectomy	GGTase-Positive Foci/cm^2 (mean ± std. error)
Experiment 1[b]			
30	10	-24 h	18.8 ± 4.6
30	10	+14 days	3.92 ± 0.91
0	10	-24 h	0.40 ± 0.14
Experiment 2[c]			
0.3	10	+14 days	1.02 ± 0.17
0.3 x 10	9	+14 days	2.16 ± 1.02
3	52	+14 days	1.51 ± 0.25
0 x 10	23	+14 days	0.59 ± 0.20

[a] Rats were administered DENA in 0.3 ml distilled water by gastric intubation. One week later the rats received 500 ppm phenobarbital in their drinking water for one week. After partial hepatectomy on day 14, the phenobarbital was decreased to 250 ppm. The rats were maintained on this concentration of phenobarbital for four to five weeks. Cyrostatic sections of liver were stained for GGTase activity and at least 2 cm^2 examined for foci.

[b] Pereira, in press.

[c] Ford and Pereira, in press.

Table 2. Possible Outcomes of a Two-Bioassay Matrix Testing of Drinking Water

	Bioassay	
Drinking Water Sample	A	B
1	+	-
2	+	-
3	-	+
4	-	-

samples 1 and 2 positive in bioassay A, and 1 and 3 positive in bioassay B. Of the three environmental samples possessing carcinogenic activity, the question then is, which sample is the most hazardous? To rank samples 2 and 3, which are positive in different bioassays, one must critically compare the results from the two bioassays. That sample 4 is negative in both bioassays does not mean it is not a potent carcinogen, unless it is demonstrated that the two bioassays as a set can detect all the carcinogens conceivably present in the environmental samples. Since most bioassays appear at least somewhat selective in their responses to well-established chemical carcinogens, it seems inappropriate to judge relative hazard on the basis of how many tests are positive.

The relative hazard of the environmental samples could be derived from the relative responses in the bioassays of the CTM, if the responses in the various bioassays were calibrated to the same standardized estimate of carcinogenic potency. A test substance would have a standardized estimate of carcinogenic potency derived from each bioassay of the CTM. A decision would have to be made on a procedure for using these individual estimates to arrive at a single estimate. This might be accomplished by averaging the individual estimates or by accepting the estimate of highest carcinogenic potency.

The following examples more explicitly illustrate the problem:

1) The Ames Salmonella mutagenicity bioassay has been proposed as correlating with carcinogenic potency (Meselson and Russell, 1977). This correlation requires the exclusion of nitrosamines, since their Ames test response is very weak compared with their carcinogenicity. When environmental samples of complex mixtures of unknown chemical composition were assayed, the Ames test would predict an erroneously low carcinogenic activity if highly active nitrosamines were present. When a liquid preincubation is used with the Ames test, nitrosamines can be detected, though still not at a level reflecting their potency. The use of liquid preincubation could also change the ranking of the other carcinogens. The results of the two bioassays (standard and preincubation Ames tests) would have to be compared as if they were two separate tests.

2) As another example, when drinking water concentrates from five cities were assayed in the Ames Salmonella mutagenicity and mouse skin initiation/promotion tests, the results of these two tests were not correlated (Loper et al., 1979; Robinson et al., 1980). The mouse skin initiation/promotion assay is very sensitive to polycyclic aromatic hydrocarbons (PAH) and nitrosamines (Pereira, in press). Comparing the responses of PAH and nitrosamines in the mouse skin and Salmonella mutagenicity tests

reveals many discrepancies (Andrews et al., 1978a, b). Among 25 polycylic aromatics, mutagenicity and carcinogenicity were positively correlated for 58% and negatively correlated for 41% (Andrews et al., 1978a). Since the two assays rank these chemicals differently, the responses of the two assays to drinking water samples are not expected to be correlated if either PAH or nitrosamines are present. The critical question is how to rank the carcinogenic activity of environmental samples based on results from more than one assay.

VALIDATION OF THE CARCINOGENESIS TESTING MATRIX

Results from two or more bioassays can be compared by calibrating each bioassay with respect to the carcinogenic potency of the chemicals to which each bioassay is sensitive. Calibration curves similar to the one used by Meselson and Russell (1977) can be determined; these investigators used the reciprocal of the log response in the short-term bioassay versus the reciprocal of the log carcinogenic potency. The reciprocal of the carcinogenic potency of each chemical can be calculated from lifetime exposure bioassay results as the dose (Dose 1/2) required to produce tumors in 50% of the animals (rats and mice) in two years. The highest estimate of carcinogenic potency is to be used in the calibration curves, since this is the value employed for extrapolation to man by the Carcinogen Asessment Group of the U.S. Environmental Protection Agency, Office of Health and Environmental Assessment.

For certain chemicals or chemical classes, individual bioassays of the CTM will respond poorly relative to the carcinogenic potencies of the chemical classes. As long as other bioassays of the CTM correctly indicate the potencies of such compounds, the responses for these compounds will be dropped from the calibration curve of any bioassay that responds poorly. For the matrix, it is the overall correlation with carcinogenic activity that is important. This method of selecting chemicals to be incorporated into the calibration curve of a bioassay results in a nonrandom grouping of chemicals, making further testing necessary for validation of the CTM.

If a chemical is extremely potent in a particular bioassay, compared with its carcinogenic potency derived from lifetime exposure, it may be necessary to delete that bioassay from the CTM. The CTM is being proposed for use with complex mixtures of undefined chemical composition and where a bioassay is not feasible. Therefore, such a response in a bioassay would tend to result in consistent overestimates of carcinogenic risk. Before a decision on keeping the bioassay in the CTM is made, the data for the chemical in the long-term carcinogenesis bioassay and the short-term bioassays will be carefully reviewed. Additional chemicals of the same general class will be tested in the bioassay to determine whether this is a general problem with the bioassay or whether it is confined to a single chemical.

Validating the CTM to rank the carcinogenic hazard of chemicals and environmental samples will involve three rounds of testing the ability of each component bioassay and of the CTM as a whole to predict carcinogenic potency. Archetypal chemicals representing the various chemical classes of carcinogens will be tested in round one to determine calibration curves. In round two, carcinogenic and noncarcinogenic (or weakly carcinogenic) analogues will be tested to determine the ability of the CTM and its component bioassay to predict relative carcinogenic potency. At this time, it should be possible to decide whether the CTM is valid and practicable and what each bioassay contributes to the CTM. The third round will involve testing additional carcinogenic and noncarcinogenic analogues, surrogate mixtures containing two or more carcinogens, and complex mixtures spiked with known carcinogens. The resulting CTM will then be ready for use in determining the relative carcinogenic hazard associated with drinking water and other environmental samples of complex mixtures.

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THE INITIATING AND PROMOTING ACTIVITY OF CHEMICALS ISOLATED FROM DRINKING WATERS IN THE SENCAR MOUSE: A FIVE-CITY SURVEY

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INTRODUCTION

Means of properly evaluating the carcinogenic risk posed by organics in drinking water are of utmost concern to the U.S. Environmental Protection Agency (EPA). While rodent lifetime exposure and human epidemiological studies serve as the only generally accepted means, the cost and time involved are highly prohibitive. In addition, formulation of human epidemiological data requires human exposure of sufficient magnitude to allow separation of confounding factors from the relationship in question. Since a major part of EPA's regulatory activities is directed towards preventing significant increases in the carcinogenic risk to the population, quick and reliable investigative methodology is a necessity. Short-term bioassays can be used to identify most potential problems and to provide an initial risk assessment.

Loper et al. (1978) showed that all the organic material isolated from the drinking water of six cities contained measurable mutagenic activity in Salmonella tester strains. These cities were selected to represent the most common types of drinking water sources. Since a large number of chemicals that are known carcinogens react positively in the Ames test, while noncarcinogens do not (McCann et al., 1975), these results suggest that chemical carcinogens are to be found in most drinking water.

Mouse skin initiation/promotion studies offer one means of confirming the presence of chemical carcinogens in complex mixtures. A positive response in this assay system may be classified as a true carcinogenic response, on the basis of

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evidence that strongly indicates a quantitative association of benign papillomas with malignant tumors (Boutwell, 1974: Burns et al., 1976; Shubik et al., 1953: Van Duuren et al., 1973). The system may therefore be used as a short-term in vivo method of assessing carcinogenic activity. Through the use of appropriate experimental designs, the system allows the activity of tumor initiators and tumor promoters to be clearly differentiated (Barenblum, 1941; Hennings and Boutwell, 1970: Mufson et al., 1977; Sivak and Van Duuren, 1971: Van Duuren, 1969).

The mouse skin bioassay was applied in our study to test both the tumor-initiating and tumor-promoting potential of a complex mixture of organic chemicals concentrated by reverse osmosis (RO) from drinking water of five cities. These samples were obtained from the same cities and processed by the same methods as employed for Ames testing by Loper et al. (1978).

METHODS

The test samples for this study were concentrated from drinking water supplies of Miami, Seattle, Philadelphia, Ottumwa (IA), and New Orleans. These cities (Table 1) were selected to represent both surface and ground types of water supply: sources potentially contaminated by agricultural runoff, industrial wastes, or municipal wastes; and uncontaminated sources (Tardiff and Denizer, 1973).

Table 1. Cities Selected for Extraction and Bioassay of Residual Organics in Drinking Water

City	Origin of Water Supply	Type of Water Supply
Miami, FL	Ground	Uncontaminated[a]
New Orleans, LA	Surface	Industrial wastes
Ottumwa, IA	Surface	Agricultural runoff
Philadelphia, PA	Surface	Municipal wastes
Seattle, WA	Surface	Uncontaminated[a]

[a] No known contamination from municipal, agricultural, or industrial wastes; however, contamination from decomposition products of natural origin is possible.

The concentrated organics were prepared for EPA by Gulf South Research Institute using the procedure described by Kopfler et al. (1977). The samples were concentrated from multiple 200-l quantities of tap water, which received sufficient concentrated hydrogen chlorine (HCl) to maintain a pH of 5.5, by RO using a cellulose acetate (CA) membrane. The reject from the CA membrane was passed through a heat exchanger to maintain the water temperature at < 15°C. Part of the reject stream was diverted through a Donnan softening unit to avoid salt precipitation. The CA permeate was adjusted to pH 10 and then concentrated by RO using a nylon membrane, and the nylon permeate was discarded. Both the CA and the nylon concentrates were then adjusted to neutral pH and extracted with pentane and methylene chloride. The aqueous phases were adjusted to pH < 2 with HCl and again extracted with methylene chloride. For the purposes of this study, these fractions were combined and the solvent removed to produce the reverse osmosis extract (ROE) sample. The residual aqueous concentrate was passed through a column of purified XAD-2 resin. After removing metallic oxides and other inorganic agents by elution with 1 M HCl and distilled water, the organics were then eluted from the column with 95% ethanol. The ethanol was removed from the eluate by vacuum distillation, and the eluates from both columns were combined to produce the XAD sample.

Once the samples were concentrated, they were administered to mice subcutaneously (into the back). The following studies were conducted using the mouse skin bioassay.

Drinking Water Concentrates as Initiators

Male SENCAR mice (sensitive to carcinogens) were obtained from Dr. T.J. Slaga, of Oak Ridge National Laboratories, Oak Ridge, TN. The mice were 8 to 10 weeks old when the study began. The ROE and the XAD samples were administered over a two-week period in six injections of 0.1 ml of a 10% Emulphor (a polyoxyetheylated vegetable oil), for a total dose of 4.5 mg/mouse (total dose = 150 mg/kg body weight). To maintain control over dosage and to allow comparison of results with prior studies (Bull, 1980), the subcutaneous route of administration was chosen. The 7,12-dimethyl benz(a)anthracene (DMBA-positive control) was given in six injections of 0.1 ml in 10% Emulphor, for a 25 μg/mouse total dose. The DMBA was obtained from Eastman Kodak Company (Rochester, NY) and was purified by thin-layer chromatography by Dr. F. Bernard Daniel of the EPA Health Effects Research Laboratory (Cincinnati, OH). There were 60 animals in each exposure group.

Two weeks after the last initiating dose, the promoting phase was begun. Forty mice of each group received 1.0 μg phorbol myristate acetate (PMA) in 0.1 ml acetone applied to the shaved back three days a week for 20 weeks. The remaining 20 animals in each group received only acetone. The PMA was obtained from Dr. Peter Borchert (University of Minnesota) and required no further purification. The animals were weighed weekly and observed for tumor incidence. The incidence of both papillomas and carcinomas was charted weekly. Any of these that persisted for three weeks or more were included in the cumulative count.

Following completion of the promotion period, the animals were held for a total of one year for study and then were sacrificed. Moribund animals were sacrificed as needed. Major organs and all macroscopically evident lesions were sectioned and fixed in 10% buffered formaldehyde solution for subsequent histopathological evaluation.

Drinking Water Concentrates as Promoters

The tumor-promoting potentials of the ROE and XAD samples were also tested in the SENCAR mouse. Groups of 20 mice (for ROE) and 30 mice (for XAD) received an initiating dose of 2.56 μg DMBA in 0.1 acetone topically to the shaved area of the back. Two weeks later, the promoting schedule with water concentrate samples was begun. The ROE from each city was applied at a dose of 100 μg per mouse per application in 0.1 ml acetone, three times a week for 18 weeks. The XAD dose was 500 μg/mouse in 0.1 ml acetone, three times a week for 18 weeks. The only reason for the differing doses was the availability of sample, which was much more limited for the ROE. A positive control group received 1 μg PMA in 0.1 ml acetone per application, following the same initiating dose of DMBA. After completing the treatment, surviving animals were held for observation of tumor incidence until they were one year old and then sacrificed. Histological evaluation was done in the same manner as in the tumor-initiating-potential study.

Drinking Water Concentrates as Complete Carcinogens

A third study was done with the concentrate samples to test their potential as complete carcinogens. In groups the same sizes as above, the ROE and XAD samples were administered topically at dose levels of 100 μg/mouse and 500 μg/mouse, respectively, in 0.1 acetone, three times a week for 20 weeks. Thereafter the same protocol was followed as in the other two studies.

RESULTS

Initiating Activity

Table 2 presents the results at the end of 50 weeks of the study testing the initiating activity of the water concentrate samples. Positive results are apparent with several of the samples. The data indicate significantly greater numbers of papillomas per mouse in the animals treated with Ottumwa ROE (0.40) and New Orleans XAD (0.33). Marginal responses occurred in Ottumwa XAD (0.28) and Philadelphia XAD (0.25). Smaller response rates were observed with Miami XAD (0.23), New Orleans ROE (0.18), and Seattle XAD (0.20), compared with the vehicle control (0.10). Essentially negative results were seen in Miami ROE (0.15), Philadelphia ROE (0.10), and Seattle ROE (0.13). Using the increase over the control response and applying a normalization factor based on the amount of water processed to obtain samples, the cities were ranked according to relative activity per unit volume. The ranking was as follows: Miami (48), Ottumwa (34), New Orleans (28), Philadelphia (26), and Seattle (7).

The time course of tumor development is presented in Figures 1 and 2. In the groups receiving ROE, no persistent papillomas occurred after week 25, except in the Ottumwa group, where the cumulative count continued to rise throughout the remainder of the 50-week period. More variation was seen in the groups receiving the XAD; in all of these groups, tumor incidence tended to increase with time relative to the control group. The New Orleans group attained the highest level of tumors per animal within the last five weeks of the study, and this change accounted for its statistically significant difference.

All lesions that were observed grossly at the time of necropsy were histologically examined; Table 3 gives the final distribution of skin tumors, but not the total count, since some lesions meeting the criteria later regressed or coalesced. The skin lesions were predominantly papillomas. As their incidence was low, the low incidence of squamous cell carcinomas observed in the experimental group was not surprising. Interestingly, the group giving rise to the most carcinomas (Seattle XAD) was negative by papilloma count. However, due to limited numbers, the incidence of these tumors did not correlate with papilloma incidence. This fact illustrates the types of problems encountered when testing relatively small quantities of complex mixtures where it is not possible to test at levels approaching a maximally tolerated dose because of sample preparation expense. Another problem was that a few fibrosarcomas were observed that could have been injection-site related.

Table 2. Cumulative Tumor Count at 50 Weeks[a]

Sample	Mice with Tumors[b]	Total Number of Tumors	Tumors Per Mouse	Normalization Factor[c]	Normalized Activity[d]
Miami					
ROE	6	6	0.15	23	1.2
XAD	8	9	0.23	360	46.8
					48.0
ROE	11	16	0.40	12	3.6
XAD	10	11	0.28	170	30.6
					34.2
ROE	4	5	0.13	14	0.4
XAD	8	10	0.25	170	25.5
					25.9
ROE	5	5	0.13	1.3	0.0
XAD	8	8	0.20	66	6.6
					6.6
ROE	5	7	0.18	10	0.8
XAD	8	13	0.33	120	27.6
					28.4
DMBA (25 µg)	38	286	7.15	-	-
Emulphor	4	4	0.10	-	-

[a] Total dose of 150 mg/kg applied subcutaneously to each animal, followed by 20 weeks of promotion with 1.0 µg PMA three times weekly.

[b] Out of a total of 40 mice.

[c] Adjustment for amount of water processed to obtain concentrates =

$$\text{normalization factor} = \frac{\text{fraction wt} \times 10^6}{\text{total recovered} \times \text{volume processed}}.$$

[d] Obtained by multiplying tumors per mouse minus control incidence by the normalization factor.

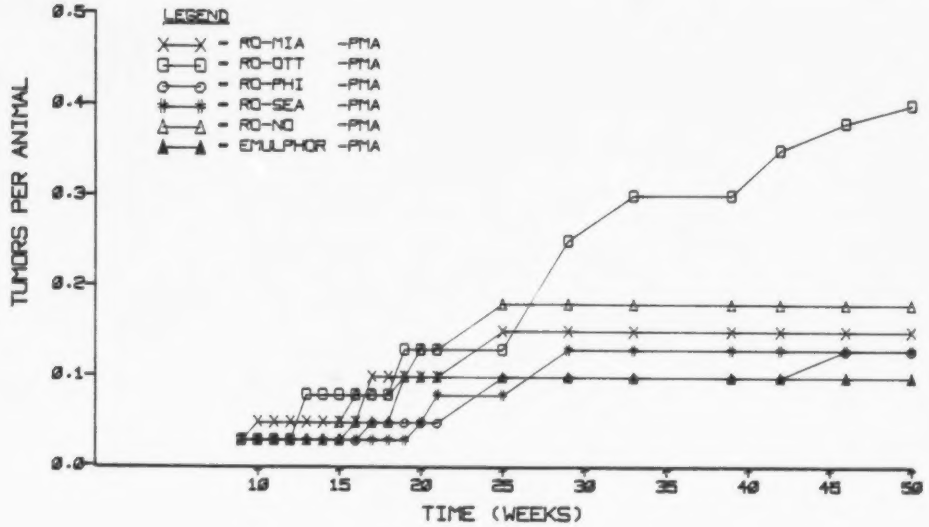

Figure 1. Tumor incidence through week 50 after animals received an initiating dose of 150 mg/kg ROE sample s.c. and PMA as a tumor promoter three times a week from week 0 to 20.

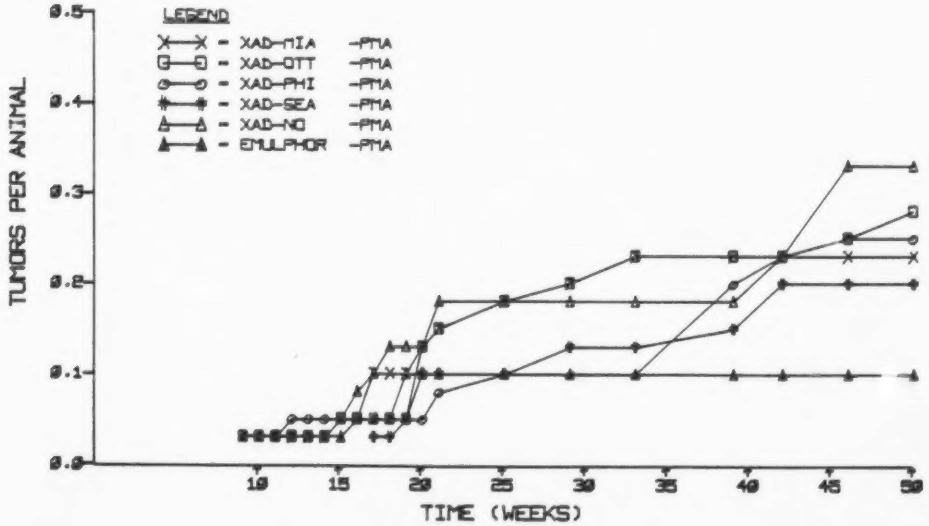

Figure 2. Tumor incidence through week 50 after animals received an initiating dose of 150 mg/kg XAD sample s.c. and PMA as a tumor promoter three times a week from week 0 to 20.

Table 3. Summary of the Macroscopically Observed Lesions

	Skin Tumors			Systemic Tumors		
Sample	Pap.[a]	Car.[b]	Fib.Sa.[c]	Pul.Ad.Ca.[d]	Hem.(li.)[e]	Hepat.[f]
Mia-ROE	4					
Ott-ROE	8			3		
Phi-ROE	2					
Sea-ROE	2					3
N.O.-ROE	2	1		3		
Mia-XAD	5					
Ott-XAD	2	1	1			2
Phi-XAD	2		1			2
Sea-XAD		2		1		3
N.O.-XAD	8	1		1	1	4

[a] Papilloma.
[b] Carcinoma.
[c] Fibrosarcoma.
[d] Pulmonary adenocarcinomas.
[e] Hemangioma (liver).
[f] Hepatoma.

The systemic tumors observed were distributed somewhat unevenly among the groups. The most frequent kind were hepatomas. Four were observed with New Orleans XAD, three each with Seattle ROE and XAD, and two with Ottumwa and Philadelphia XAD. Animals treated with the other four samples gave no evidence of hepatomas, and only one animal in the control group gave evidence of hepatomas. Pulmonary adenocarcinomas were also somewhat elevated in New Orleans and Ottumwa ROE samples. Due to the relatively low incidence of these tumors, the differences could not be considered statistically significant. However, incidences in experimental groups generally exceeded those observed in control animals.

Promoting Activity

The study to determine the promoting potential of the water concentrate samples is continuing, and Table 4 shows the results through week 38. The groups receiving 500 μg XAD sample per application from Miami, New Orleans, and Ottumwa have yielded one papilloma each. No papillomas have occurred in groups treated with

Table 4. Drinking Water Concentrates as Promoters: Number of Papillomas Per Number of Mice[a]

			City				
Sample	Vehicle	PMA	Mia.	N.O.	Ott.	Phil.	Sea.
Controls	0/20	319/20					
ROE (100 μg)			0/20	0/20	0/20	0/20	0/20
XAD (500 μg)			1/20	1/30	1/30	0/30	1/30

[a] Initiator equals DMBA (2.56 μg/mouse applied topically). Drinking water concentrate or PMA (1.0 μg in 0.1 ml acetone) applied topically three times a week for 18 weeks. Results at 38 weeks.

Philadelphia and Seattle XAD, nor have papillomas occurred in any of the ROE samples. In the positive control group, using 1 μg PMA three times weekly, 19 of 20 mice had tumors, with a total of 319 papillomas. At this point, it appears that drinking water samples at the doses applied do not promote DMBA tumorigenesis.

Complete Carcinogenic Activity

Table 5 gives the results after 38 weeks of studying the potential of the ROE and XAD samples as complete carcinogens. Only one papilloma has been observed, and that is in the New Orleans ROE group. To this point in time, no evidence of complete carcinogenesis of organic chemicals from drinking water has been demonstrable. Again, the chemicals isolated from drinking waters do not seem to be complete carcinogens at the doses applied.

DISCUSSION

Within the limits of possible dosage in the present work, it appears that tumorigenic and/or carcinogenic substances were present in the drinking waters. These chemicals were primarily initiators in the mouse skin rather than promoters or complete carcinogens. However, this conclusion must be clearly couched in terms of the doses that were actually administered. Compared with PMA, organic chemicals present in the ROE and XAD fractions were less than 1/100 and 1/500 as potent as promoters, respectively.

Table 5. Drinking Water Concentrates as Complete Carcinogens: Number of Papillomas Per Number of Mice[a]

Sample	City				
	Miami	N.O.	Ottum.	Phila.	Seattle
ROE (100 mg)	0/20	1/20	0/20	0/20	0/20
XAD (500 mg)	0/30	0/30	0/30	0/30	0/30

[a] Drinking water concentrate applied topically three times a week for 20 weeks. Results at 38 weeks.

In view of the extreme potency of PMA, this was not altogether a satisfying result. A further reservation is that no evidence exists to indicate that mouse skin is a universal target tissue for tumor promoters.

In the case of the Ottumwa ROE sample and all of the XAD samples, tumor development was late. This contrast with the time course of tumor development for the positive control DMBA (Figure 3) suggests that the chemicals in drinking water responsible for initiating tumors may differ from DMBA with respect to underlying mechanism(s). In terms of initiating activity, individual samples produced significant increases in the number of tumors. At equal doses of organic material, however, there was little to distinguish positive from negative responses in the different samples. On the other hand, if the data were adjusted to the amount of water processed, the total units of activity present could vary among water samples by a factor of seven. Although this calculation was based on somewhat nonsignificant data, it does suggest that the total risk observed might parallel the level of organic material present. Undoubtedly, a wide variety of variables underlie this parallel. For example, previous work has shown that disinfecting drinking water can increase the levels of carcinogens isolated from drinking water (Bull, 1980). However, the observation argues that a prudent course of action in drinking water treatment might involve reducing the total organic carbon present in the finished drinking water. Although the data obtained in the present study cannot be used to estimate risks to populations consuming these drinking waters, it certainly justifies further research into carcinogenic risks associated with drinking water.

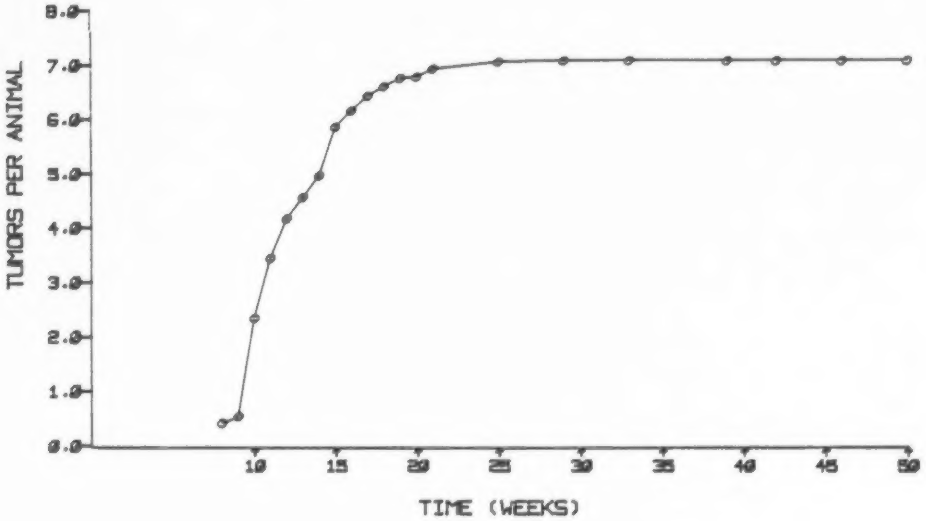

Figure 3. Tumor incidence through week 50 after animals received an initiating dose of 25 μg DMBA s.c. and PMA as a tumor promoter three times a week from weeks 0 to 20.

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AQUEOUS EFFLUENT CONCENTRATION FOR APPLICATION TO BIOTEST SYSTEMS

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INTRODUCTION

Potential chemical mutagens in industrial effluents may be present at concentrations below the detection limits of biotests such as the Ames mutagenicity test. These chemicals may accumulate in biological food chains. Many insecticides and other chemicals are known to accumulate in living organisms where tissues act as effective storage depots for toxic compounds (Loomis, 1978). This effect is especially significant for human health when dilute toxicants enter the human food chain, such as through seafoods. Mollusks such as the oyster tend to accumulate toxicants, because they filter-feed, which concentrates and magnifies the effects of toxic materials. Because of this potential for bioaccumulation, methods are needed to determine the bioactivity of low concentrations of potential toxicants in industrial effluents.

The objective of the research program discussed in this paper was to evaluate and compare three methodologies for concentrating potential chemical mutagens in typical industrial effluents for application to *in vitro* biotest systems. For this study, the Ames *Salmonella* mutagenicity assay (Ames et al., 1975) was used. The optimum concentration methodology would ideally meet the following criteria:

1) concentration of relatively large quantities (> 3 l) of aqueous sample;

2) concentration factors of ≥ 200 times;

3) little or no loss of volatile compounds;

4) efficient concentration and extraction of potential toxicants;

5) maintenance of high integrity of chemicals by preventing artifact formation;

6) maintenance of the relative concentrations of all compounds;

7) use of methods and reagents that are compatible with the biotest system; and

8) maintenance of microbial sterility of the resulting sample.

This paper describes the experimental approach and resulting data for three concentration methods: adsorption using macroreticular resins (XAD), freeze-drying (lyophilization), and reverse osmosis (ultrafiltration). These methods were used to concentrate added standard chemical compounds (i.e., potential toxicants) in "typical" aqueous effluents for application to the Ames mutagenicity test.

METHODOLOGY

Three methods were used to concentrate aqueous effluents for application to *in vitro* biotest systems: sorbent extraction, lyophilization, and reverse osmosis. A schematic is presented in Figure 1.

Five-gallon samples of raw wastewater obtained from an industrial plant served as typical standard effluent samples and were used to evaluate each of the concentration methodologies.

To check for microbial contamination, aliquots of the neat effluent samples were streaked with a sterile applicator onto both Difco Bacto nutrient agar and Ames histidine-free bottom agar. The plates were highly contaminated, indicating the need for filter sterilization. The samples were sterilized by drawing them through a series of Millipore filters of 1.3-, 0.45-, and 0.2-μm pore size. The Ames *Salmonella* mutagenicity assay (Ames et al., 1975) was used to test the neat filtrate for mutagenicity, using two histidine-requiring strains, TA98 and TA100. The specific procedure for analyzing the neat effluents used five concentrations in triplicate: 0.01, 0.1, 0.5, 0.75, and 1.0 ml/plate. Each concentration was tested with and without rat liver S-9 fractions in the plate-incorporation test. Spot and toxicity tests were also

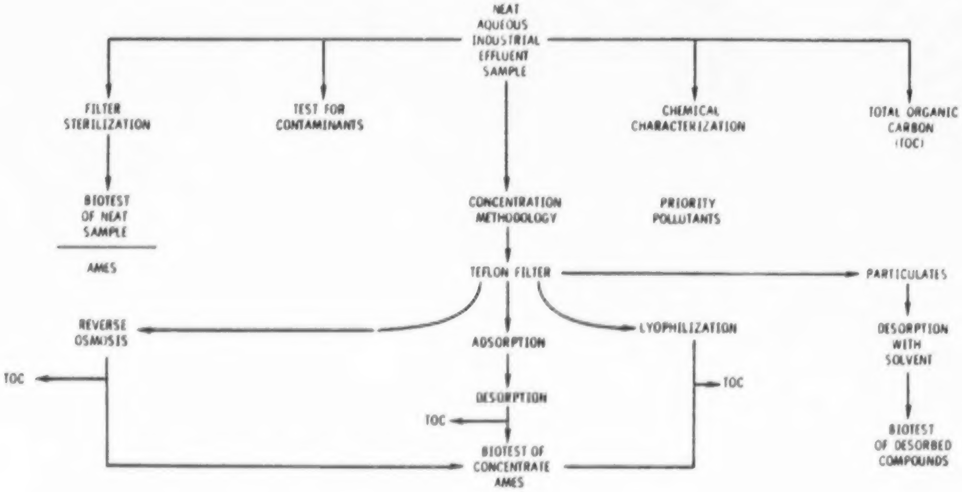
NEAT AQUEOUS INDUSTRIAL EFFLUENT SAMPLE
FILTER STERILIZATION
TEST FOR CONTAMINANTS
CHEMICAL CHARACTERIZATION
TOTAL ORGANIC CARBON (TOC)
BIOTEST OF NEAT SAMPLE
AMES
CONCENTRATION METHODOLOGY
PRIORITY POLLUTANTS
TEFLON FILTER
PARTICULATES
REVERSE OSMOSIS
ADSORPTION
LYOPHILIZATION
DESORPTION WITH SOLVENT
TOC
DESORPTION
TOC
TOC
BIOTEST OF CONCENTRATE AMES
BIOTEST OF DESORBED COMPOUNDS

Figure 1. Schematic of concentration and biotesting of textile-industry effluents.

performed. The positive controls used were 2-nitrofluorene (2NF), sodium nitrite, 9-amino-acridine, benzo(a)pyrene (B[a]P), and 2-amino-anthracene, while tap water served as a negative control.

The concentrates from sorbent extraction and lyophilization were tested using six concentrations in which the samples were not toxic: 0.1, 0.4, 2.0, 10, 30, and 100 µl/plate. If the samples were found to be toxic at a designated concentration, a lower, nontoxic concentration was used as the highest concentration.

Many effluent samples contain large amounts of particulate material that impede flow through XAD resins, reverse osmosis membranes, and sterilizing filters. The large particles (> 5 µm) in the effluent used in this study were removed by filtration. Potential toxic and mutagenic compounds adsorbed to particles were also removed. These compounds were assayed for bioactivity. The industrial effluent was filtered under vacuum through polyester drain discs placed in series with 5-µm Teflon type LS Millipore filters. Twenty-four-hour Soxhlet extractions were carried out on filter blanks with four fresh polyester drain discs and six 5-µm Teflon type LS Millipore filters, using 200 ml of methylene chloride. A cellulose extraction thimble retained the filters. The methylene chloride was reduced to 5 ml with a Kuderna-Danish concentrator over a steam bath. Five milliliters of dimethylsulfoxide (DMSO) was added, and attempts were made to remove the rest of the methylene chloride; however, approximately 6 ml of organic extract remained, indicating that approximately 1 ml of methylene chloride would not evaporate. The material was transferred to a micro-Snyder apparatus for more efficient removal of methylene chloride; however, the volume still was not reduced. The presence of residual methylene chloride was confirmed by infrared spectrophotometry.

Ames mutagenicity testing of the Soxhlet filter extract of recovered particulates indicated no mutagenicity, but a slight toxic response was found. A Soxhlet reagent blank was also tested and gave a slight mutagenic response in the spot test and a toxic response. A methylene chloride control gave both a mutagenic and a toxic response. Although there was no clear mutagenic response for the Soxhlet filter particulate extract, methylene chloride should not have been used in processing samples for biotest systems because of its potential for causing a false mutagenic response. Acetone or other nonbioactive solvents are recommended for future Soxhlet extractions.

Three known mutagenic materials were used in this evaluation: acridine orange (AO), B(a)P, and 2NF. All are positive mutagens in the Ames microbial test system. AO, a precursor to some dyes which might be found in textile effluents, is a highly colored compound. AO was used first in all of the experiments because it could be easily traced by visual methods. B(a)P is a chemical mutagen commonly found in environmental samples. This compound requires S-9 activation and also represents less-stable compounds. It is light sensitive and presents some concentration problems. A commonly used positive standard in the Ames test is 2NF, which does not require activation. The selection of nonmutagenic concentrations of standard compounds for spiking the neat effluent sample was based on previous experimental data (unpublished). All of the concentrations of positive mutagens added to the neat effluent theoretically should be mutagenic when concentrated by at least a factor of 200 times. The spiked concentrations of mutagens prior to concentration are listed in Table 1.

The standard EPA method (EPA, 1977) was used for chemical characterization of these selected priority organic compounds. This procedure involves solvent extraction and gas chromatography/mass spectrometric (GC/MS) analysis of the effluent. The GC/MS system used was a Hewlett-Packard 5985A GC/MS/Data System. Total organic carbon (TOC) analyses were performed on the neat standard effluent samples and on the concentrates to determine efficiencies of recovery of organic compounds. The TOC analysis was performed on a Technicon II Autoanalyzer using the TOC cartridge at 550 nm; the recorder read full scale at 200 ppm.

Table 1. Standard Mutagenic Compounds and Concentrations Added to Effluent Samples

Compound	Concentration (mg/1)
Benzo(a)pyrene	0.17
Acridine orange	7.60
2-Nitrofluorene	0.17

Sorbent Extraction

Sorbent materials have been used primarily to remove organic compounds from potable water whose matrix is relatively clean (Junk et al., 1974; Loper et al., 1978). More recent investigations (Rappaport et al., 1979) have used macroreticular resins such as Amberlite (Rohm and Haas) to remove potential mutagens from effluents and wastewaters. Compared with drinking water, industrial effluent samples are usually higher in particulates, contain larger numbers of much higher concentrations of organics, and have more extreme pH values. Processing waste effluents with XAD resins presents additional problems not encountered in the treatment of drinking water. Considerably more research is required with the various sorbent materials to determine the optima of parameters such as depth of bed, flow rates, desorption methods, and solvents, as well as breakthrough limits. Such studies would be highly complex because of the different interactions of each chemical compound with the sorbent materials.

The adsorbent used in this study was Rohm and Haas Amberlite XAD-2, a low polarity styrene-divinylbenzene copolymer possessing the macroreticular characteristics necessary for high sorptive capacity. Recovery efficiencies of about 80 organic compounds in water have been reported (Junk et al., 1974). The efficiencies vary from 35 to 100%; on average, however, recovery efficiencies are above 78%.

The initial XAD-2 (Applied Science) column was prepared by washing the resin with a 50:50 mixture of methanol and water and pouring off the excess solvent to leave a slurry of XAD-2. The system consisted of a 300-ml burette with a plug of silanized glass wool placed in the bottom to retain the particulate XAD resin. The XAD-2 slurry was added to the column, and a glass wool plug was placed on top of the slurry to prevent disruption of the XAD particulates forming a column of XAD-2 resin 2 cm long by 1 cm in diameter. The resin bed was washed with 3- to 30-ml aliquots of deionized water and maintained wet at all times.

Three liters of raw wastewater effluent was processed by filtering the sample through a 5-μm type LS Millipore filter to remove coarse particulates. This filter became plugged after 200 ml of sample was processed, and a pre-filter consisting of a Unipore polyester drain disc (Bio-Rad Laboratories) was placed ahead of the 5-μm Millipore filter. This modification allowed filtration of 600 to 700 ml of sample before the system became plugged with particulates. The 3-l sample was subsequently processed in 600-to 700-ml aliquots. Filters were replaced whenever plugging occurred. The filters with particulates were retained for solvent extraction of organics. After about half of the effluent had been processed, the flow rate slowed considerably, and highly purified nitrogen under pressure was applied to facilitate the filtering process. The average flow rate was about 1.2 l/h.

Direct extraction with DMSO was evaluated as a means of reducing experimental time by eliminating desorption with one solvent followed by exchange with DMSO. Three milliliters of DMSO were added to the XAD column, saturating the XAD resin. The solvent was permitted to stand for 30 min. Then the DMSO solution was drained into a sterile test tube.

Unconcentrated raw wastewater was filter-sterilized by passage through a series of Millipore filters, as described earlier. Ames mutagenicity and toxicity tests were also performed. The sample was found to be nonmutagenic and nontoxic. The DMSO-extracted XAD-2 concentrate solution was then tested for mutagenicity. The concentrated effluent processed through the methanol/water-washed XAD-2 columns indicated some Ames mutagenicity bioactivity, but no dose response. The XAD-2-processed control tap water gave a similar response.

A new XAD-2 column was prepared by the method of Junk et al. (1974), whereby three solvents (methanol, acetonitrile, and diethyl ether) are used to wash the resin in a Soxhlet extraction apparatus. The XAD resin was refluxed with each solvent for 8 h and then stored in methanol. A 3-l tap water blank was processed with washed XAD-2 and then tested for mutagenicity. No mutagenicity was found. Three liters of effluent were concentrated on the washed XAD-2; again, no mutagenicity was found.

We concluded from this study that 1) the neat effluent sample and the 600-fold concentrate were nonmutagenic; 2) DMSO extracts were marginally mutagenic if the XAD-2 was washed only with methanol/water; and 3) DMSO could be used directly as a desorbent solvent if the XAD-2 were washed properly (i.e., by the Junk method).

Standard mutagens were added to the standard effluent in order to determine the efficiency of recovery and to evaluate the mutagenicity of the concentrate using the Ames test system. The three standard mutagens were AO, B(a)P, and 2NF.

The zinc chloride salt of AO was made up by adding 22.8 mg AO to 3 l of particulate-free raw wastewater. This solution, containing 170 ppb AO, was processed (concentrated by adsorption) by the procedure described previously. The flow rate through the XAD averaged 18 ml/min. The AO content of the effluent was measured by a colorimetric technique with an Aminco DW-2 dual wavelength UV-Vis spectrophotometer scanning the range of 400 to 650 nm. The wavelength monitored was 433 nm. The adsorption efficiency for AO was determined by comparing the concentration prior to processing with that of the XAD-2 filtrate. The starting material (neat effluent) contained 7.6 µg/ml, and the filtrate contained 4.5 µg/ml, indicating a collection efficiency of 40.8%. Desorption was achieved by adding 5 ml of DMSO for a residence time of 30 min. A comparison of the amount of AO collected (9.3 mg) with the desorbed amount in the DMSO (7.5 mg) indicated a desorption efficiency of 80.6%. Mutagenicity was tested by applying the unconcentrated effluent containing 7.6 mg/l (7.6 ppm) AO to the Ames plate-incorporation test. No mutagenicity was found. The final XAD-2/AO extract, concentrated from 3 l of starting sample to 5 ml of DMSO (with a combined 32.9% recovery and desorption efficiency), contained 1.5 mg/ml (1,500 ppm). The total concentration factor was 196. A dose response was found using TA98 with S-9.

Five hundred micrograms of 2NF was added to 3 l of particulate filtered sample effluent, making a concentration of 167 µg/l (167 ppb). The XAD-2 was prepared, as described previously, with three solvents in a Soxhlet system. Three liters of sample effluent containing the 167 ppb 2NF was processed. The recovered 2NF was desorbed with 5 ml of DMSO. Mutagenicity testing indicated no response in the Ames test to the blank or to the neat, unconcentrated sample. A positive dose response with TA98 and S-9 and a response to TA100 (no dose response) was found for the XAD-2 concentrate.

Addition of 500 µg of B(a)P to 3 l of neat effluent gave a concentration of 167 µg/l (167 ppb). The 3 l of neat effluent was concentrated by passing it through the XAD-2 column. Five milliliters of DMSO was used to desorb the recovered B(a)P. The blank sample and the neat sample containing 167 ppb B(a)P gave negative responses in the Ames test. The concentrated B(a)P sample indicated bioactivity in tests performed on two different dates: on 7/13 at 30 µl/plate with TA98 and S-9 (with no dose response) and on 7/19 at 30 and 40 µl/plate (with no consistent dose response). Recovery experiments were performed to determine extraction efficiency of B(a)P by XAD-2 from spiked effluent with subsequent desorption with DMSO. Much care was taken to isolate all samples containing B(a)P from light in order to eliminate potential light-degradation problems. Five milligrams of B(a)P was added to 3 l of industrial effluent, and the sample was processed through the XAD-2 column. Recovery efficiencies were determined by measuring the B(a)P solutions with an Aminco DW-2 dual wavelength UV-Vis spectrophotometer. The aqueous permeate was analyzed at 270 nm by scanning the range of 230 to 410 nm. The UV analysis showed that no B(a)P passed through the XAD-2, indicating a possible 100% recovery of B(a)P. The column was desorbed with 5 ml of DMSO. The UV analysis indicated a desorption efficiency of 45% of the B(a)P. This low desorption efficiency may indicate a permanent bonding of the B(a)P to the XAD-2 resin. The UV data gave no evidence of degradation of the B(a)P.

Lyophilization

This freeze-drying approach to concentration is best applied to effluents that contain water-soluble, nonvolatile, heat-labile pollutants. Inorganic salts and biological compounds of large molecular weight are retained by this method. Bieri et al. (1979) have reported successful use of freeze-drying to remove water from Chesapeake Bay samples for application to chemical characterization tests. These researchers reported the potential loss of volatile compounds below C_{12} hydrocarbons and problems with chemical contamination from vacuum-pump oils. Van De Meent et al. (1977) suggested that freeze-drying led to catalytic conversion of alcohols to olefins. However, Bieri found no evidence of this problem (Bieri et al., 1979). In the present study, contamination in lyophilized samples was demonstrated. Consequently, all samples had to be filter-sterilized before treatment or after concentration prior to adding them to the Ames test system.

In this experiment, the lyophilization system was built around a stainless steel drum manifold (Virtis Model 10-MR-ST). The other components were a vacuum pump, a backup trap, a vacuum gauge, and the sample-containing filter-seal flask. A vacuum of 0.133 mbar was maintained over processing times of 48 to 50 h. The system could run unattended during much of this time, including overnight. The backup trap retained much of the volatile material and could be analyzed for volatile compounds.

Three liters of neat effluent sample was processed to dryness, leaving 0.8 g of a dry white residue. An inorganic chemical characterization analysis (EDAX) indicated the presence of sulfur, silicon, potassium, calcium, and iron. TOC analysis of the residue using the Technicon II Autoanalyzer, was 6.3%. This dry residue was dissolved in 10 ml of 50:50 DMSO:water solution. A concentration factor of 300 times was achieved. Problems were encountered in completely dissolving the residue. A "dissolved" sample was applied to the Ames mutagenicity assay using the plate-incorporation test and indicated no mutagenicity in the lyophilized neat effluent. Microbial contamination was encountered, but not enough to prevent a valid test. Filter sterilization is recommended prior to lyophilization.

AO was added at a concentration of 0.0076 mg/ml to 3 l of effluent sample. The sample was lyophilized in approximately 55 h. Using the colorimetric method, 96.3% of the AO was retained in the lyophilized sample. The freeze-dried powder was dissolved in a 50:50 mixture of sterile distilled water and DMSO and applied to the Ames mutagenicity assay. A two-point dose response was obtained at 10 μl and 2 μl with TA98 and S-9 activation. Microbial contamination was so high that the plates had to be hand counted; lower concentrations could not be counted because of the contamination.

Reverse Osmosis

Reverse osmosis (RO) has become a versatile separation and purification method. This process physically separates contaminant from water by circulating the aqueous solution at high pressures over the surface of a semipermeable membrane. Two factors influence the concentration of contaminants: the physical and chemical properties of the contaminants and the properties of the membrane. Recent RO technology has improved rapidly as membrane technology has advanced. Much of the development and applied research has been directed toward the purification of aqueous effluents, whereas this study is concerned with the concentrate.

The RO system used in this investigation was manufactured by Abcor, Inc. (Cambridge, MA). The system is a bench-type static RO and ultrafiltration test cell designed specifically for studying membrane selectivity in a laboratory situation. The RO unit has a 200-ml capacity, maximum operating pressure of 105 kg/cm (gauge), and a membrane diameter of 3 in. (7.6 cm). It is 6.25 in. high by 3.75 in. in diameter (15.9 x 9.5 cm) and weighs 6 lbs (2.7 kg). The stainless steel unit has a Teflon-coated magnetic stirring bar for constant agitation at the membrane surface and uses a SEPA-97 (Osmonics) cellulose acetate membrane of nominal 5-Å (0.5 nm) pore size.

Three liters of raw wastewater was filtered to remove particulates to prevent clogging of the RO membrane. A Unipore polyester drain disc (Bio-Rad Cat. No. 334-0659) was placed over a type LS Millipore filter with a 5.0-μm pore size. The filtered effluent was spiked with AO to obtain a concentration of

0.0076 mg/ml. The spiked effluent was processed by RO. A processing time of about 48 h was required to reduce the sample to 168 ml. Problems were encountered with a much-reduced flow rate toward the end of the processing experiment. The RO unit was dismantled and a large buildup of what appeared to be inorganic materials highly colored with AO was found on the membrane, impeding flow through the membrane. The retained solution apparently became saturated with both dissolved salts and AO as the concentration increased; this phenomenon will limit the concentration factor attainable by RO. The efficiency of concentration was tested colorimetrically: the concentration of AO in the starting unprocessed spiked sample was 7.6 mg/ml and in the processed concentrate 7.1 mg/ml. A conductivity measurement of the filtrate (EP Meter, Myron L Co.), reduced from 3 l to 200 ml, indicated that 77% of the conductivity was removed by the process. This result is an obvious limitation of the RO system.

CONCLUSIONS

This investigation has demonstrated that an industrial effluent spiked with subtoxic amounts of standard mutagens (AO, 2NF, and B[a]P) can be concentrated by XAD-2 resin. The work also showed that AO could be concentrated by lyophilization and RO. The other two mutagens were not tested with the latter two techniques. The concentrated spiked effluent exhibited positive dose responses in the Ames *Salmonella* biotest. The RO technique was found to retain 84% of the TOC. However, only a tenfold concentration was achieved, because of clogging of the membrane by precipitated compounds.

This evaluation also showed that the adsorbent concentration procedure has the following advantages over lyophilization and RO techniques: low cost of equipment, short processing time, ease of portability, and potential for selectivity for specific chemical classes by resins. A disadvantage of this approach is the need for a desorption solvent, which increases the chances of sample alteration, chemical contamination, loss of sample, and possible lack of compatibility with biotest systems.

The lyophilization procedure has the advantage of good recovery of inorganic components and relatively high organic compound recovery. However, it requires much processing time, and the equipment costs are high.

Reverse osmosis also concentrates inorganic components. It is portable and has the potential advantage of recovering specific chemical classes of compounds by use of selective membranes.

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SESSION 3

TERRESTRIAL SYSTEMS

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POTENTIAL UTILITY OF PLANT TEST SYSTEMS FOR ENVIRONMENTAL MONITORING: AN OVERVIEW

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Research over the past decade has shown a significant proportion of genetic diseases in man to be caused by natural and man-made chemical mutagens. Human cancer is one of the diseases for which direct associations with certain environmental factors have been established (such as the association between cigarette smoking and lung carcinoma). The recently published "Atlas of Cancer Mortality" (Mason et al., 1975) provides further evidence for the association between human cancer and environmental factors. In this study, certain specific types of cancer appear to be associated with certain industrial activities. Chemical mutagens are also believed to contribute to birth defects, behavioral abnormalities, and aging. It has been suggested that environmental chemicals play a role in causing atherosclerosis (Benditt, 1973) and, most damaging of all, in deteriorating the human gene pool.

Awareness of the role of environmental chemicals as human health hazards has led to the development and use of various techniques for identifying potentially toxic chemicals and, if possible, eliminating exposure to them. These methodologies include several nontraditional types of short-term bioassays. A variety of test systems, ranging from the use of viruses to that of circulating human lymphocytes and sperm cells, have been used to identify potentially harmful chemicals or mixtures of chemicals. Over 100 different assays have been developed for detecting toxic chemicals (Hollstein et al., 1980).

Despite their historical role in the formulation of principles of genetics and genetic toxicology, plant test systems have not been employed in the recent rapid advances in the development of genotoxin-detection technology. This general neglect may perhaps

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be attributed to the apparently great phylogenetic distance of plants from animals. However, the use of plants as test organisms for detecting the genetic effects of individual chemicals or as monitors of environmental quality offers the following advantages:

1) Plants, like animals, are eukaryotes; thus, their organization and cell structure are similar.

2) Plants undergo mitosis and meiosis, thus making it possible to evaluate somatic and germ cell mutations and their transmission to future generations. This capacity is especially important when we consider that most of the short-term bioassays use bacteria or mammalian cells in culture, which do not undergo meiotic cell division.

3) Plants can easily be propagated from vegetative tissue or even from a single cell. Thus any variant line can be genetically characterized.

4) Plants show a wide array of genetic endpoints, including gene mutation, DNA repair, primary DNA damage, and chromosomal aberrations. In certain plant species (e.g., barley and *Arabidopsis*), multiple-locus forward-mutation test systems have been developed that may be particularly relevant to human genetic systems. Using *Arabidopsis*, it is already feasible to monitor one hundred loci at once. This attribute is significant when we consider that different chemicals elicit qualitatively and quantitatively different responses at different loci in the same genome and that most of the commonly used short-term bioassays monitor genetic alterations at only one or two loci.

5) Plants are relatively easy and inexpensive to work with. Furthermore, mutational events are very easily scored by technicians.

6) Perhaps the most significant attribute of plants as test systems is their suitability for *in situ* monitoring.

For the last three years, the U.S. Environmental Protection Agency (EPA) and the National Institute of Environmental Health Sciences (NIEHS) have made concerted efforts to develop and use plant bioassays for environmental monitoring. There are two areas in which plant systems show promise for immediate use: 1) as part of a short-term first- or second-level laboratory test battery for evaluating the mutagenicity of specific environmental chemicals or chemical mixtures, and 2) in field monitoring studies, as indicators of the mutagenicity of the total environment.

Possible Role of Plants for Mutagenicity Evaluation in the Laboratory

A number of articles have been published emphasizing the need for short-term bioassays (see Hollstein et al., 1979, for a review) and for their integrated use with *in vivo* animal bioassays in identifying environmental chemicals and estimating risk from exposure to them (Waters et al., 1980). An extensive data base is not yet available for comparing the genetic responses to chemicals of plant test systems with those of other *in vitro* and *in vivo* animal test systems. However, preliminary comparisons based on review of the literature show a fairly good concordance. The genetic potencies of eight chemicals in various *in vitro* and *in vivo* test systems were compiled by Clive and Spector (1978). The data in Table 1 show that mutation responses in plants correlate well with those in mammalian systems.

Table 1. Comparative Mutational Potencies of Eight Chemicals in Bacteria, Plants, Insects, and Mammals[a]

Chemical	Bacteria	Plants	Insects	Mammals *in vivo*	Mammals *in vitro*
Trenimon	2	1	4	1	4
Mitomycin C	4	2	1	1	2
MNNG	5	3	7	5	2
Triethylene-melamine	8	4	8	3	5
Ethylenemelamine	3	5	3	4	8
Ethylmethane sulfonate	7	6	6	7	3
Methylmethane sulfonate	1	7	2	8	7
Dimethyl-nitrosamine	6	8	5	6	6

[a]Data from Clive and Spector (1978).

Rédei et al. (1980) have compiled data from the literature on the mutagenic response of the multilocus *Arabidopsis* test system to a number of known animal carcinogens. The list includes several compounds that require metabolic activation to produce genetic effects. This report shows an 84% correlation of genetic responses in Arabidopsis with those in animal tests. It also shows that Arabidopsis can provide the enzymes needed to transform promutagens into reactive metabolites. Because it is a multiple locus system and because Arabidopsis has a short generation time and can grow on a variety of media, this test seems especially well suited for further development for screening environmental chemicals in the laboratory. This is the only existing short-term bioassay for analyzing the genetic effects in progeny of environmental exposure of the parents. The EPA, in a cooperative effort with the University of Missouri, is currently validating this assay.

Nearly every biology student first visually encounters chromosomes in onion or broad bean root tips--the chromosomes from these materials are large and easy to manipulate. A few chemical pesticides have been tested for their ability to induce chromosomal aberrations in plant root tips; Table 2 compares the clastogenic response to these pesticides in plant root tips with that in mammalian cells in culture.

Table 2. A Comparison of Responses by Plant Root Tip Cells and Mammalian Cells in Culture to Pesticides[a]

	Chromosome Aberrations	
Compound	Plant Root Tips	Mammalian Cells in Culture
Apholate	+	+
Atrazine	+	+
2,4-D	+	+
DDT	+	+
Dichlorvos	+	+
Dieldrin	+	+
Ethylene dibromide	-	-
Griseofulvin	+	+
Hempa	-	-
Heptachlor	+	+
Maleic hydrazide	+	-
Mercury compounds	+	+
Phosphamidon	+	+
2,4,5-T	+	+
Tepa	+	+

[a]Data from W. F. Grant (1978).

In general, the positive or negative responses to these pesticides are very similar in plant root tips and mammalian cells in culture. The only exception seems to be maleic hydrazide. It has recently been observed by Dr. Michael Plewa (University of Illinois, personal communication, 1980) that although animals cannot transform this compound to genetically reactive metabolites, plants (at least *Zea mays*) do convert it into biologically active forms.

The Health Effects Research Laboratory of EPA at Research Triangle Park, NC, is in the process of evaluating the *Vicia faba* root tip assay for possible inclusion in the level one test battery. Several pesticides for which we have fairly extensive data will be tested for chromosomal effects in this assay.

The presentation by Dr. Constantin in this symposium (Constantin et al., 1980) further illustrates the utility of plant cytogenetics assays in concert with microbial mutagenicity assays for evaluating the potential health effects of complex environmental mixtures. With few exceptions, plant bioassays do not have as well-defined gene markers or genetically engineered tester strains as are found in microbial bioassays. On the other hand, very few microbial assays can be used to evaluate the chromosomal effects of exposure to environmental chemicals.

The point of this discussion is that plant bioassays could be profitably used for toxicological evaluation of environmental chemicals. These assays will not be able to replace microbial test systems in the foreseeable future, but will be useful in furnishing complementary information.

The Role of Plants for In Situ Environmental Monitoring

Perhaps the most useful testing application for plants in the future will be in monitoring the mutagenicity of the total environment. Environmental chemicals exert their effects not in isolation but in concert with other chemicals and environmental factors. This environmental milieu is impossible to reproduce in the laboratory. By growing experimental plants in the ground at the site to be tested, one can evaluate the multimedia exposure effects. Several plant systems have shown a great deal of promise for *in situ* environmental monitoring. The *Tradescantia* stamen hair assay (Schairer et al., 1978) has been used to monitor ambient air quality at several industrial sites in the U.S. The waxy pollen maize assay (Plewa, 1978) has been developed and applied to detect the mutagenicity of agricultural chemicals. Klekowski (1978) has developed a very useful bioassay for monitoring mutagens in effluent streams, rivers, and lakes. An excellent review of these test systems has been edited by de Serres (1978).

In the present symposium, Dr. Ma describes the potential use of a newly developed bioassay for monitoring complex environmental mixtures (Ma et al., 1980). The *Tradescantia* micronucleus assay appears to be even more sensitive than the *Tradescantia* stamen hair assay. This assay is under further validation with financial support provided by EPA.

Another assay, developed by Dr. Vig, of the University of Nevada (Vig, 1980), uses a soybean system for measuring point mutations and chromosomal aberrations in somatic cells. By using a series of promutagens (compounds that require mammalian metabolic activation enzymes for biotransformation to express their genetic potential), Dr. Vig has shown that plants have the ability to activate these compounds to mutagenic levels.

In the past, the lack of concurrent controls has caused some difficulties in interpreting data from *in situ* bioassays. Data from historical controls cannot be used as a substitute for on-site controls. *In situ* monitoring with plant test systems is not intended to take the place of more rigorous testing to evaluate the health hazards of exposure to a particular environment. None of the *in situ* plant bioassays have reached a stage of development where they could be used to identify specific mutagenic compounds from the environment. Their main utility so far appears to be in raising a "red flag," so that priorities can be set for applying more specific bioassays and chemical analysis to track down the sources of toxic chemicals.

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ARABIDOPSIS ASSAY OF ENVIRONMENTAL MUTAGENS

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INTRODUCTION

In the past, Arabidopsis assays have been employed for testing the mutagenic effects of a variety of chemicals. Although it is potentially useful for determining mutagenic hazards of complex mixtures, this species has not been much used for this purpose.

An Arabidopsis assay system was initiated in the early 1960's by Andreas Müller (1963, 1965b). The suggested procedure was based on the principles first exploited by Gregor Mendel in his famous pea experiments. In the autogamous species of pea, within individual flowers, segregation of the alleles at meiosis and the random combination of the gametes at fertilization resulted in the reappearance of both dominant and recessive phenotypes of the parents among the embryos developed on the F_1 plants. Therefore when Mendel crossed two different varieties of peas (Yellow vs. green and Smooth vs. wrinkled cotyledons), a study of only the F_1 plants, bearing the F_2 seeds, was frequently satisfactory for his genetic analyses. Thus, he saved considerable time and labor, enabling him to make more comprehensive studies.

But heterozygosity within the nucleus of a cell can also arise by mutation. Mutation from a dominant to a recessive allele in the diploid cells is concealed. If the mutation takes place early in the diploid germline, and the heterozygous cell gives rise to a sector encompassing both the pollen-producing (androecium) and the egg-producing (gynoecium) lineages, segregation may become evident among the embryos of the same individual. Since the germline is commonly multicellular, the F_1 plant may be chimeric after a mutational event. This is more of an advantage than a disadvantage because it permits the screening of a large population at a low cost.

Such an analysis is impractical, however, in monoecious plants, such as maize, because the silks and the tassel may not differentiate concordantly (from the same cell lineage). Obviously, dioecious plants and the majority of bisexual animals are not amenable to such an analysis.

PURPOSE

Though a large number of assay systems are available for mutagens, none of them suits entirely the needs for testing all hazardous compounds. The most efficient and the most widely used microbial assays involve the detection of revertants at a specific locus. The mutability of individual loci may vary by one or more orders of magnitude (Table 1). Furthermore, the reversion assay uses special alleles, one or another at a time. The mutability of these special sites depends to a great extent on the nature of the inducing agent (Table 2). Therefore it is not easy to draw firm conclusions as to how complex mixtures of mutagens or even pure compounds would affect other genes. Even worse, the mutability of many important human genes cannot be directly measured, and the inferences based on indirect methods are also somewhat tenuous. Approximations based on mammalian assays, such as the mouse specific locus assay (which is probably the best), are not much better either, because we do not know for sure whether the six or seven loci used represent accurately all the loci of mice (or those of man).

Table 1. Rates of Spontaneous Mutation[a]

Organism	Mutant Phenotype	Rate
E. coli	Streptomycin resistance	4×10^{-4}
	Histidine auxotrophy	2×10^{-6}
Neurospora	Inositol independence	2×10^{-5}
	Adenine independence	4×10^{-6}
Drosophila	Yellow body	1×10^{-4}
	White eye	3×10^{-5}

[a] Rédei, 1980, p. 576.

Table 2. Site Specificity of Induced Mutations in Yeast[a]

	Revertants per 10^7 Cells			
Sites	EMS[b]	NMG[c]	UV[d]	γ-rays
cyl-131	1226	1775	41	18
cyl-133	4	1	83	8
cyl-9	8	8	2430	19

[a] Abridged from Prakash and Sherman, 1973.

[b] Ethylmethane sulfonate.

[c] Nitrosomethylguanidine.

[d] Ultraviolet.

There is obviously a need for improvement in the testing systems: we must search for approaches capable of detecting a variety of genetic alterations (gene mutations and chromosomal effects) at as large a number of positions in the genome as possible. We must also strive to learn much more about the capabilities of metabolism in activating or detoxifying the potentially hazardous chemical compounds present in the human environment. The tests must be relatively fast, reliable, reproducible, and inexpensive, and the information obtained should be applicable for predicting human hazards. The Arabidopsis assay outlined here appears quite valuable for meeting many of these goals.

THE ARABIDOPSIS ASSAY

Culture of the Plants

Arabidopsis thaliana (L.) Heynh. is a plant of the family Cruciferae (mustards), with a haploid chromosome number of five. The early genotypes may produce eight generations a year in the laboratory, where the life cycle can be hastened by continuous illumination (long-day plant). Under short daily light regimes, the vegetative period is quite long, and only a few generations can be grown in a year. Under such conditions, the plants grow much larger and may produce 50,000 seeds each.

The plants can be grown in pots on any good soil or other media. In the greenhouse, we culture them on soil in 5-in (12.7-cm) pots, each with several plants. In growth chambers, we have successfully raised 200 or more plants on Pro-Mix medium (Premier Brands) to maturity in petri plates 10 cm in diameter.

Before planting, the medium was moistened with a nutrient solution of the following composition:

ammonium nitrate (NH_3)	200 mg/l
magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	100 mg/l
calcium phosphate, monobasic ($CaH_4(PO_4)_2 \cdot H_2O$)	100 mg/l
potassium phosphate, monobasic (KH_2PO_4)	100 mg/l
potassium phosphate, dibasic (K_2HPO_4)	50 mg/l
ferricitrate	2.5 mg/l

After germination, the lid was removed, and the lost moisture was replaced by distilled water as needed (at least two times a day). In soil culture, supplemental nutrients are not needed. In any case, the seeds should not be covered after planting, because *Arabidopsis* requires light for germination.

When large quantities of seeds need to be planted, time may be saved by suspending the seeds in a fluid yet viscous agar solution (ca. 0.15%) and spreading them dropwise with the aid of a separatory funnel or a pipette. After planting, the seeds must not be allowed to dry for any length of time. To avoid washing them to the rim of the container, watering is best done initially with a fine mist. Later, after germination, the pots can be placed in a tray containing distilled water. In the growth chamber, the intensity of the light should not exceed 800 foot-candles (8608 lux), and a constant temperature of about 24°C is satisfactory.

The seeds of *Arabidopsis* loose their germination ability within two to three years at higher temperatures and humidity. Therefore, it is advisable to store the seeds under a regime where the sum of the temperature in degrees Fahrenheit and the relative humidity is below 100: the lower this figure, the longer the viability.

Mutagen Assay

Generally, for laboratory assays, seeds are exposed to mutagens. Though the mature embryo contains about 6000 to 7000 cells, only two of the cells represent the diploid germline at this stage. As the seeds germinate and the seedlings develop, the number of cells in the germline increases. The consequences of mutation at two different stages of the growth of the germline are diagrammed in Figure 1.

Either the mutants can be detected at the embryo stage (Figure 2) or the M_2 generation can be planted and classified for seedling and plant traits. The most convenient method of assessing the mutagenic effects is to determine the frequency of mutational events expressed at the embryo stage. For this purpose the fruits

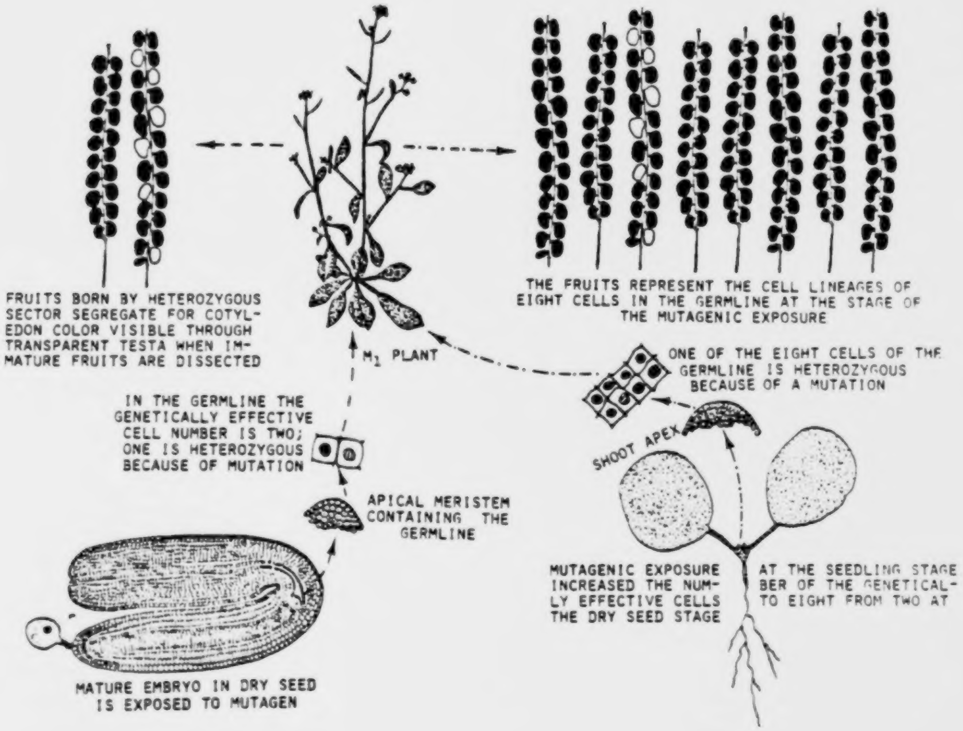

FRUITS BORN BY HETEROZYGOUS SECTOR SEGREGATE FOR COTYLEDON COLOR VISIBLE THROUGH TRANSPARENT TESTA WHEN IMMATURE FRUITS ARE DISSECTED
THE FRUITS REPRESENT THE CELL LINEAGES OF EIGHT CELLS IN THE GERMLINE AT THE STAGE OF THE MUTAGENIC EXPOSURE
M_1 PLANT
ONE OF THE EIGHT CELLS OF THE GERMLINE IS HETEROZYGOUS BECAUSE OF A MUTATION
IN THE GERMLINE THE GENETICALLY EFFECTIVE CELL NUMBER IS TWO; ONE IS HETEROZYGOUS BECAUSE OF MUTATION
SHOOT APEX
APICAL MERISTEM CONTAINING THE GERMLINE
MUTAGENIC EXPOSURE AT THE SEEDLING STAGE INCREASED THE NUMBER OF THE GENETICALLY EFFECTIVE CELLS TO EIGHT FROM TWO AT THE DRY SEED STAGE
MATURE EMBRYO IN DRY SEED IS EXPOSED TO MUTAGEN

Figure 1. Consequences of mutagenic treatment at stages when the germline consists of two diploid cells (left) and when it has increased to eight (right). Explanations are on the diagram.

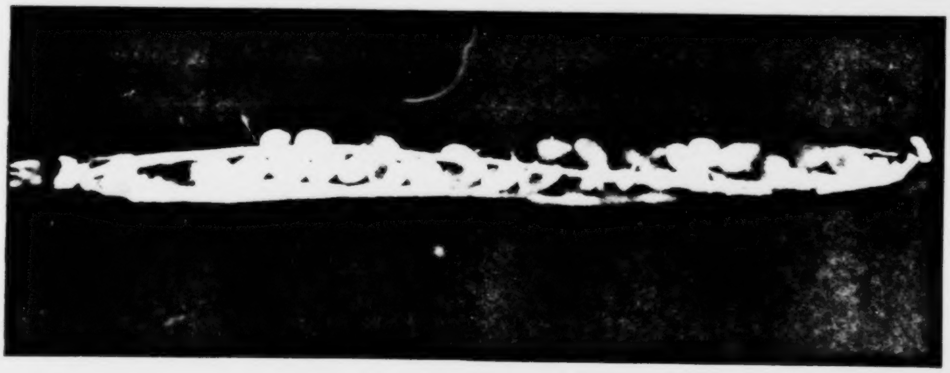


Figure 2. An Arabidopsis fruit, showing segregation for the color of the embryos.

are opened with sharp forceps before the seed coat turns brown and opaque. Through the immature seed coat, the green cotyledons of the normal embryos can be seen. Many types of mutants have white, yellow, or pale green cotyledons, which can also be classified at this stage (Figure 2). Persons with very good vision may not need a magnifier; if necessary, an enlarger should be used which does not interfere with the use of the hands. With a little experience, approximately 70 fruits can be screened within an hour.

Organization of the Germline

The fruits appear on the stem in a rather well-organized pattern (Figure 3). The arrangements (phyllotaxis) follows a spiral, and after some turns, two or more fruits appear on the same vertical line. It is expected that the vertically aligned fruits belong to the same cell lineage. In chimerical plants, the genetic constitution of the same lineage is expected to be the same, while other cell lineages may be different (Figure 1) indicating that the germline is composed of two cells in the mature seed. (This statement will be supported later.) If one of these cells contains a mutation, the plant is expected to have two sectors, one of the normal constitution (homozygous wild type) and another that is heterozygous for the mutation. The fruits situated on the stem may represent one or the other sector. Though the periodicity of the fruit arrangement on the stem may be influenced to some extent by developmental factors or by the external conditions, the experimenter must be familiar with the phyllotaxis in order to make the work efficient. As a rule of thumb, it is advisable to scan fruits which sit on opposite sides of the stem, as these will most likely represent different sectors of the chimera. It is possible, however, for phenotypically identical mutants to occur in opposite fruits; these, most likely, originated from independent mutational events (Figure 4).

Calculation of the Mutation Frequency

Within single fruits, the segregation of mutant and wild-type embryos is expected to correspond to monogenic Mendelian ratios. Dominant and recessive mutations may be distinguished if the number of embryos is sufficiently large. Single fruits may contain up to 30 or 40 embryos, though frequently their number is much reduced when the chemicals are toxic. Occasionally, recessive mutations may mimic ratios expected on the basis of dominant inheritance, because of the small numbers and/or reduced transmission of the chromosomes (gametes) involved. The simultaneous induction of two phenotypically similar mutations may result in a theoretical proportion of 9 wild-type:7 mutant. Poor transmission of the chromosome carrying the wild-type allele (because of a large

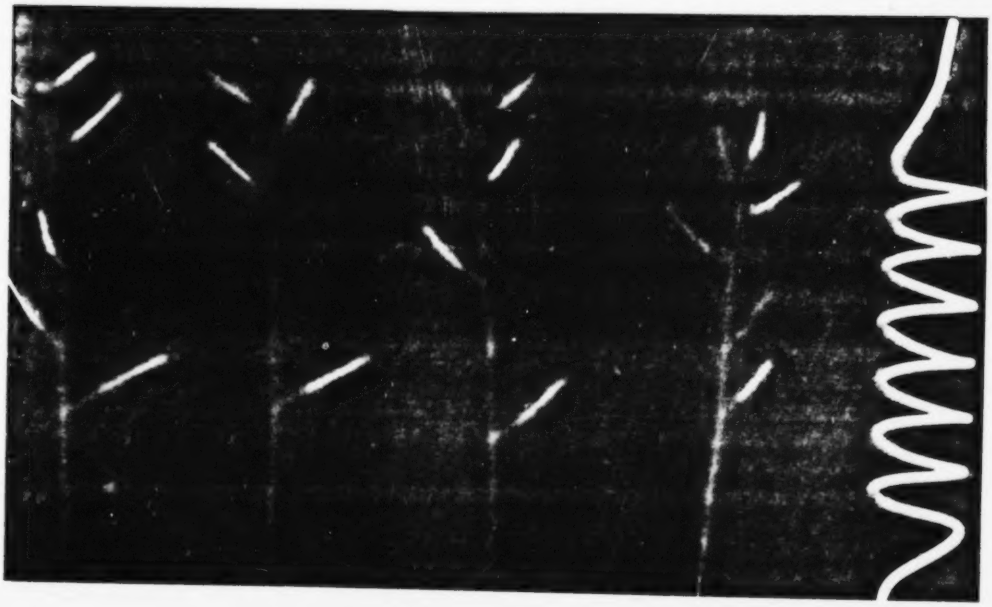


Figure 3. Arabidopsis main stem showing the phyllotaxis of the fruits (in a right-hand spiral, as illustrated at right).

deficiency or other gametophyte factors) may give a 2:1 ratio. It is sometimes not possible to distinguish clearly among these proportions on the basis of the few embryos in a fruit.

In addition, we may observe a deficiency of the mutant class: this situation is actually the most common. The reduction of the mutant class within a fruit can be caused by the poor transmission of the chromosome carrying the mutant allele or by the early elimination of some of the zygotes homozygous for defective alleles.

When there is normal inheritance of the mutants and not too high a frequency of mutation (i.e., the majority of plants would incur only a single mutation), we can determine rather accurately the number of sectors in the mutant plants. Alternatively (or additionally), we may harvest all the seeds from individual M_1 plants and determine segregation ratios in the planted M_2 generation. In the case of normal transmission and viability, the proportion of the wild-type and mutant individuals in the progeny is expected to reflect the number of cells in the germline that gave rise to the fruits. (This is what we call the genetically effective number of cells, or GECN.) Where the germline is

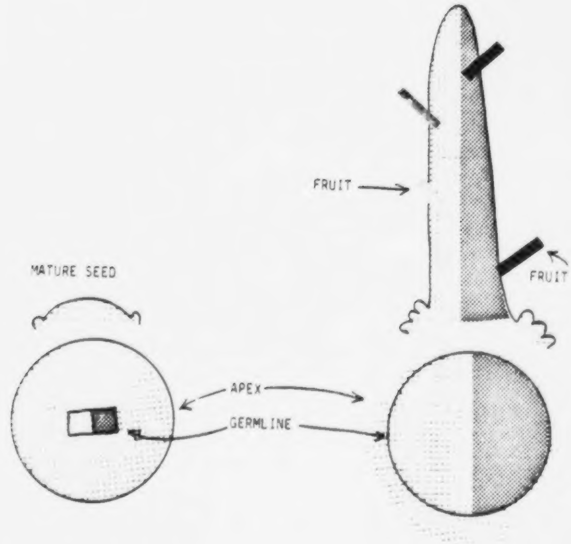


Figure 4. The time course of the development of the germline. In the mature embryo. the germline consists of about two cells (left): in the maturing plant, the number of cells in the germline increases (right). If one of the two cells of the germline in the mature seed incurs a mutation, the growing plant becomes a chimera containing two identical-size sectors. The genetic constitutions of the fruits (embryos) are then determined by their locations on the stem.

represented by a single cell at the time of the mutation, segregation for wild-type and mutant should show a proportion of 3:1 (GECN = 1). Where the germline contains two cells (GECN = 2) at the time of the mutagenesis and only one of the two cells suffers a mutation, the expected segregation ratio is 7:1: that is, one of the two cells segregates 4:0 while the other displays 3:1 proportions, and pooling the data, we obtain the 7:1 ratio. Similarly, if the germline were eight cells, seven of the fruits would be expected to yield only wild-type embryos, and one would display 3:1 segregation progeny, a proportion of 31:1 ([7 x 4] + 3 : 1).

Thus, on the basis of genetic data, we can infer the number of genetically effective cells at the time of the mutagenic exposure if it is of relatively short duration, that is, lasting for a few hours rather than for many days. The number of genetically effective cells is reasonably consistent at a particular developmental stage. Poor sampling of the seed output of the plants and reduced transmission of certain mutants may, however, cause variation around the most frequent class (Figure 5.)

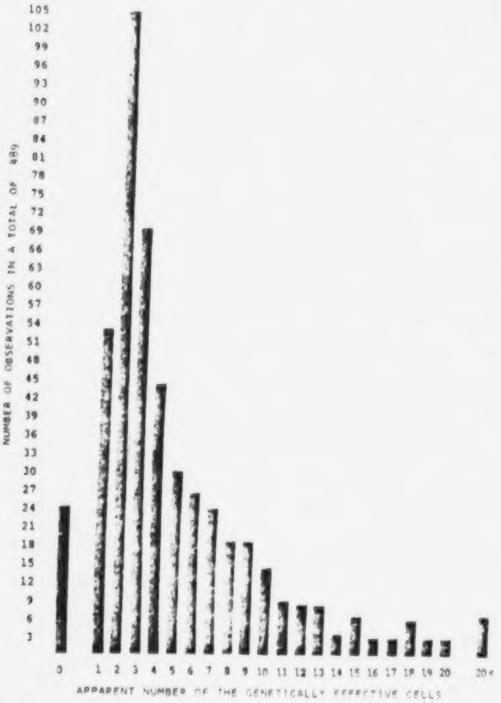


Figure 5. Variations in the apparent number of cells in the germline. The mode (the most frequent class) indicates that the number of genetically effective cells (GECN) is generally two. The classes shown were established by grouping the segregation data of all mutants classified and counted.

When the number of genetically effective cells is known, the frequency of mutation can be easily calculated by the following formula:

$$R = \frac{\text{no. of independent mutations observed}}{\text{no. of progenies classified x GECN x ploidy x dose of mutagen.}}$$

This formula expresses mutation frequency on a genome basis, because we take into consideration the diploid nature of all the cells in the germline by substituting 2 for ploidy in the denominator. By this procedure, mutation frequencies can be directly compared with those of other higher organisms or microoganisms. The dose of the mutagen used may be omitted from the formula.

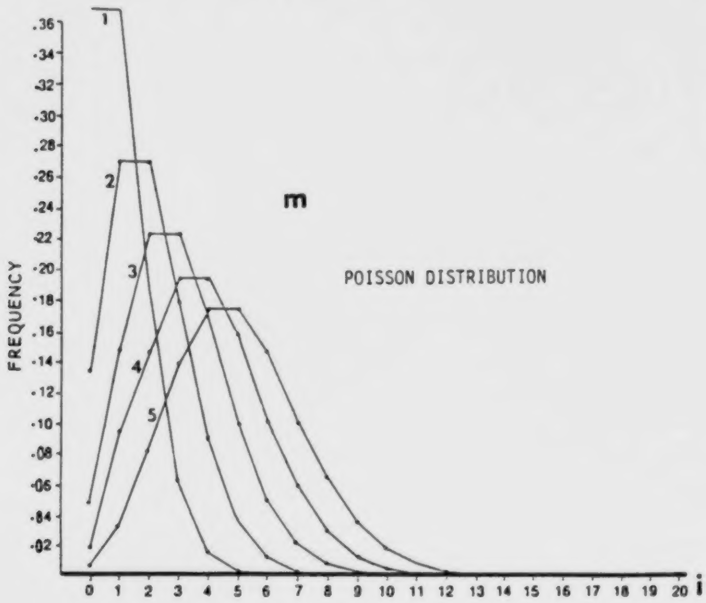
In an experiment, we treated mature seeds with 0.3% ethylmethane sulfonate, and the embryos were classified before maturity as outlined (Table 3). The fruits not used for embryo analysis were harvested, and in the M_2 generation, phenotypically distinguishable mutant classes were counted in 308 families. Some of the families displayed no mutants at all, and others showed one or more types. The frequency distribution of these families is shown in Figure 6, with the theoretical expectation based on the Poission distribution.

Table 3. Frequency of Embryo Mutants after Mutagenic Treatment with 0.3% Ethylmethane Sulfonate for 15 Hours

Treatment	No. of Plants	No. of Genomes	No. of Fruits Analyzed	Mutation Frequency
Untreated	192	768	599	0.0013
Treated	205	818	485	0.4707

This analysis considered all the mutants that germinated and expressed themselves during early or later stages of development. Many mutations that could be identified in the immature fruits could not be detected after planting the seeds. Apparently, these involved lethality that prevented germination of the individuals affected. A number of additional types of mutants could, however, be identified during the later stages of development.

A closer examination of the classes of families with various or no mutational events revealed some similarities with and some discrepancies from the Poisson distribution. Curiously, the frequency of families with one or more mutations was higher than



308 Families with Number of Phenotypically Distinguishable Mutations	0	1	2	3	4	5	6
Number Observed	22	115	118	45	6	2	0
Frequency	0.071	0.373	0.383	0.146	0.020	0.007	0

Figure 6. Comparison of the distribution pattern of families with multiple mutations with Poisson distributions for averages of one to five independent events (m). The abscissa (i) represents the numbers of events expected to occur at the frequencies given by the ordinate.

expected on the basis of the curves shown. These curves show Poisson distributions with 1, 2, 3, 4, and 5 average mutational events expected per family. On the other hand, the frequencies of classes 1 and 2 were nearly equal, a feature characteristic of the Poisson distributions of integer numbers (Figure 6, top).

In the 308 families, 520 mutations were identified on a phenotypic basis. This classification was obviously loaded with a systematic error, because in the presence of two independent mutations per cell, we would also expect, besides the two single mutants, the double-mutant class. In the case of three mutational events, three single mutants, three different types of double mutants, and one triple-mutant phenotype may be exhibited in a family. Since progeny analysis could not be performed with the lethal or subvital types, the genetic constitution of these phenotypic classes could not be determined.

The average number of mutational events per family can be determined, however, if we consider the zero-mutation class. The frequency of this group is not influenced by the complications just mentioned. The theoretically expected frequency of the zero-mutation class (based on the Poisson distribution) was determined for 2.0 to 3.0 mutational events per progeny, and the frequencies are shown in Table 4.

Table 4. Theoretically Expected Frequency of the Zero-Mutation Class

Average no. of mutations	2.0	2.5	2.6	2.7	3.0
Expected frequency of zero-mutation class	0.135335	0.082085	0.074274	0.067206	0.049787

Figure 6 (bottom) shows the result of an experiment where the frequency of the zero-mutation class was 0.071, which indicates an average of 2.6 to 2.7 mutations per family (Table 4). Since at the time of the treatment with EMS, the germline of each mature embryo contained four genomes, the average frequency of mutations in this material can be computed as (approximately) 2.6/4 = 0.65. This is considerably higher than the observed 0.47 for mutations expressed at the embryo stage in the immature fruits; it is also higher than the empirically found value of 0.59 for all the mutations observed. We may also conclude that under the conditions of this experiment, approximately 10% of the mutations were missed, either because of misclassification or due to some other accidental causes. The difference between the experimentally observed value of 0.59 and the frequency of 0.65 predicted on the basis of Poisson's exponential binomial limits is not so large as to give basis for serious reservations concerning validity.

Calculation of the Number of Target Loci

From the viewpoint of effectiveness of a mutagen assay, it is important to know how many potential targets can be hit by a mutagenic chemical. It seems that the mutability of the gene loci is not uniform across the entire genome (Table 1). This may be due to differences among the various loci in number of nucleotide pairs (the size of the genes). The genes may be more mutable during periods of replication or transcription, and these differences in state may be reflected in the observed mutabilities. Also, certain sites within a gene may appear as "hot spots" when exposed to one agent but not when another mutagen is applied (Table 2). The nature of particular base pairs and/or the conformation of the DNA may also affect mutability. The metabolic machinery, the genetic repair systems, etc., may also affect the various loci differently.

Assays that are capable of monitoring mutational responses at a large number of loci, presumably representing all of the loci in a fair manner, are particularly attractive. The number of genes cannot be directly counted in higher organisms. Since nucleotide sequencing became practical, the number of genes of a few viruses could be determined (Fiers, 1975; Sanger et al., 1977). Such an approach is still impractical for mammals or higher plants, which may have six to eight orders of magnitude more DNA per cell than the smallest viruses.

The number of genes (cistrons) in Drosophila is believed to be about 5,000 (Garcia-Bellido and Ripoll, 1978), a figure that is close to the number of bands detectable by the most revealing counts on the salivary gland chromosomes. Belling (1928) assumed that the number of chromomeres observed in the lily chromosomes, 2193, indicates the number of genes in this plant. Because of the small size of the chromosomes of Arabidopsis, chromomeric organization cannot be determined (Figure 7), but even if this plant were quite favorable for cytological studies, an estimate of the number of genes on such a basis would not be sufficiently realistic.

The number of gene loci can be calculated more precisely on the basis of overall mutation frequencies if the average rate of mutation per locus is known. In Arabidopsis, estimates of mutation rate are available for specific loci involved in the synthesis of thiamine (vitamin B_1). For the calculations, mutations induced with EMS at the three loci and in the following numbers were used: py 35, tz 5, th 11. The average induced mutation rate at these three loci is 3.2×10^{-5}, which is comparable to the data for mice from the literature (Table 5).

These loci of Arabidopsis were chosen for studies not on the basis of their mutability, but because of our interest in the genetic control of a biosynthetic path. They may represent to a

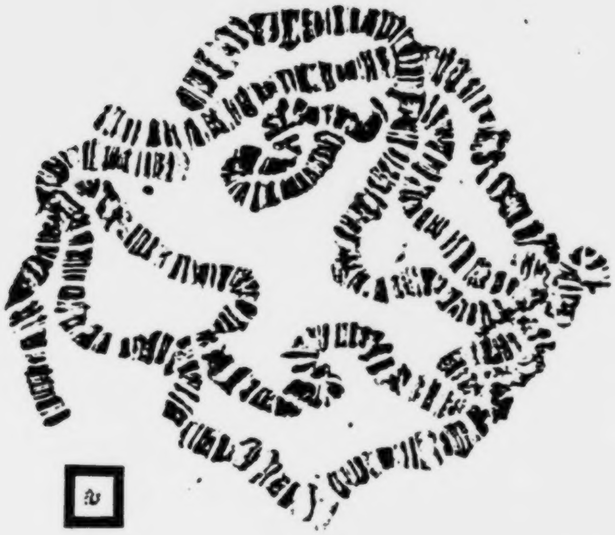


Figure 7. Salivary gland chromosomes of Drosophila compared on an equal scale with the chromosomes of Arabidopsis, shown within the box. (Courtesy of Dr. H.K. Mitchell and Dr. Lotti Sears, respectively.)

Table 5. Induced Mutation Rates in Arabidopsis and Mouse

Organism	Loci	Mutagenic Agent	Rates
Arabidopsis	py	(EMS)	2.0×10^{-4}
	tz	(EMS)	2.5×10^{-5}
	th	(EMS)	7.0×10^{-5}
Mouse	Isozyme loci	X-rays[a]	1.7×10^{-4}
	7 Specific loci	EMS[b]	7.8×10^{-5}

[a] Malling and Valcovic, 1978.
[b] Ehling, 1978.

fair extent the rest of the genes of this plant. This assumption cannot be proven, however. In the absence of better information in this or other systems of higher eukaryotes, we have no other choice.

When the estimated overall frequency of induced mutations is divided by the average frequency of mutations per gene, we obtain an estimate of the number of loci capable of mutation or of the expression of a mutant phenotype at a particular developmental stage. For example, the average frequency of mutations detectable at the embryo stage was found to be approximately 0.47 (Table 3), and the average rate of mutation per locus was estimated to be 3.2 x 10^{-5}. Hence, the minimal number of loci responding with mutations expressed at the stage of immature embryos is 0.47/3.2 x 10^{-5} = 14,687. Similarly, on the basis of mutations expressed at other developmental stages, we can calculate the number of genes expressed in mutant states at early, late, or all developmental stages (Table 6).

Table 6. Calculation of the Number of Loci in Arabidopsis

		Frequencies
Embryo mutations per genome		0.47
Early seedling mutations per genome		0.32
Late seedling mutations per genome		0.12
Frequency of all mutations observed	0.47 + 0.12 =	0.59

Average mutation frequency of 3 loci: 3.2 x 10^{-5}

Estimate of the number of loci with embryo mutations:

$$\frac{0.47}{3.2 \times 10^{-5}} = 14{,}687$$

Estimate of all the potentially mutable loci (based on Table 4 data):

$$\frac{0.65}{3.2 \times 10^{-5}} \times 20{,}313$$

Estimate of the number of all loci with mutations observed:

$$\frac{0.59}{3.2 \times 10^{-5}} = 18{,}438$$

Some objections may be raised to this procedure of calculating gene numbers. For example, these average mutation frequencies may include repeated mutations at some loci and none at others. The repeated occurrences do not seriously bias the data, however, because the probability of including twice a locus with a high, say 10^{-2}, frequency of mutation is only 10^{-4}. Such cases do not much affect the average frequencies, which are in the 10^{-1} range. Thus the expected error in connection with each case is in the 0.0001 range. Similarly, the very stable loci barely bias the figures because the use of approximation shown in Table 5 affords the proper correction.

Since this mutagen test assesses forward mutations, the actual number of sensitive sites substantially exceeds that of the number of genes. If we assume that an average locus consists of one thousand nucleotide pairs, the number of potentially mutable sites per plant may reach several millions.

Critical Population Size

The potencies of various mutagens are very unequal, yet we must evaluate their effectiveness in a reliable manner. Therefore some guidelines are needed as to the size of the populations to be tested in order to find some mutants even if their expected frequency is very low. Also, we must establish some criteria of clearance for an apparently innocuous compound. We need to know how many plants to screen to find at least one mutant or what number of mutations represents a significant increase over the spontaneous rate.

The critical size of a population can be defined as the number of plants to be tested, or better, the number of genomes, which may yield at least one mutation at a chosen level of probability (P). The rationale of the procedure is that we rule out the chance of finding no mutational events at all more frequently than specified by 1-P. Let us use a very simple example: When a plant is heterozygous for a single allelic pair, according to the Mendelian rule, we expect 3/4 of the progeny to have the dominant phenotype and 1/4 of the individuals to display the recessive genotype. How many individuals (n) do we then need in the M_2 generation in order to find at least one recessive plant with a probability (P) of 0.99? Since $P = 1-(3/4)^n$,

$$n = \frac{\log (1-P)}{\log (3/4)} = \frac{-2}{-0.1249387} = 16.008 \approx 17.$$

Similarly, if we expect an induced frequency of mutations of 1/200 genomes, the number of genomes to be tested (n) at P = 0.99 should be

$$n = \frac{\log (1-P)}{\log (199/200)} = \frac{-2}{-0.002177} = 918.7$$

to find at least one genome within the germline with a recessive mutation at the 0.99 level of probability. When we treat mature seeds, where the germline is represented by four genomes (as we discussed earlier), we need to test (918.7)/4 = 230 surviving plants.

The question remains whether an "induced" frequency of mutations is higher than the spontaneous rate. Certainly we must use a negative control. The frequencies between the two groups should be compared by determining the appropriate confidence limits. We must remember that even if no mutation is observed in 100 genomes, this may not negate the possibility that we missed up to four according to the 95% confidence limit. Similarly, we may want to know whether the 10% mutations observed among 200 genomes is significantly different from the 15% observed in 100 genomes. Reference to the 0.95 confidence belt convinces us readily that they are not. Were we to have 25% mutations in the latter group, the difference would be significant at the 0.95 level.

CORRELATION BETWEEN MUTAGENIC EFFECTS IN ARABIDOPSIS AND CARCINOGENICITY IN AMIMALS

The embryologist Theodore Boveri attributed cancer to the presence of an abnormal complex of chromatin as early as 1914. Evidence for and against a genetic cause of cancer has been entertained and occasionally negated ever since. In the widely used Ames *Salmonella* assay system, of more than 200 carcinogens over 90% showed mutagenic effects (Hollstein et al. 1979). A survey (Rinkus and Legator, 1979) of 465 known or suspected carcinogens examined by the *Salmonella* S-9 method indicated a lower correlation (77%) with mutagenicity. It seems that not all groups of carcinogens are equally efficient mutagens for all organisms.

With *Arabidopsis* so far, approximately 110 chemical compounds had been tested for mutagenicity, according to a survey of about four dozen publications. Interestingly, this compares favorably with *Drosophila*, for which about 1000 publications are listed by the Environmental Mutagen Information Center as being concerned with tests of about the same number of compounds (Hollstein et al., 1979).

We have found information concerning carcinogenicity and neoplastic effects for 52 compounds tested for mutagenicity in *Arabidopsis* (Rédei et al., in press). The correlation between carcinogenicity in animals and mutagenicity in *Arabidopsis* seems comparable to or better than that for *Salmonella* (Table 7). It is particularly noteworthy that only one compound, di-2-chloroethylamine phosphamide ester (endoxan, cytoxan), was negative in the mutagenicity assay among those that are listed as category I carcinogens for animals (proven in at least two animal systems) according to the Occupational Safety and Health Administration (OSHA). For this particular carcinogen, only a short negative note is available concerning mutagenicity in *Arabidopsis* (Müller, 1965a), which may require revision on repetition. This compound (synonym cyclophosphamide), when administered to male mice, showed dominant lethal effects for spermatozoa, but no dominant lethals were induced when spermatocytes or spermatogonia were tested (Röhrborn, 1970). According to Heddle and Bruce (1977), this compound is carcinogenic and shows positive responses in the mouse sperm abnormality, bone marrow micronucleus, and *Salmonella* tests. Triethylenemelamine is another false negative in *Arabidopsis*; it was found to be mutagenic in several other assays, including the sex-linked recessive lethal test in *Drosophila*, in the mouse specific locus test, and in a *Salmonella* assay (Hollstein et al., 1979). Again, the negative result in *Arabidopsis* is found in the same undetailed note (Müller, 1965b).

Table 7. Number of Compounds Tested for Mutagenicity in *Arabidopsis* and for Carcinogenicity in Animal Assays

	Carcinogens	Neoplastic
Mutagenic in *Arabidopsis*	40 (87%)	4 (66.6%)
Nonmutagenic in *Arabidopsis*	6	2
Total	46	6

OSHA Categories of Compounds That Are Nonmutagenic in *Arabidopsis*	
Di-2-chloroethylamine phosphamide ester	I
Ethyl alcohol	II
Chloramphenicol	II
Maleic acid hydrazide	III
Sulfathiazole	III
Triethylenemelamine	?

The remaining four nonmutagenic suspected carcinogens are only category II and III compounds, indicating that the evidence for carcinogenicity is fragmentary or insufficient. I was unable to find positive mutagenic information for them in the commonly used mutagenic assay systems.

CONCLUSIONS

The Arabidopsis assay as outlined provides direct evidence concerning the genetic nature of most of the mutagenic alterations. The majority of the mutants detected can be subjected to formal genetic analysis. This system permits the simultaneous study of forward mutation at thousands of loci; therefore, the information obtained must be relevant for practically all the genes of this organism and presumably for other eukaryotes. Because of the very small size of the chromosomes of Arabidopsis, cytological proof for chromosomal aberrations is hard to obtain (except for some very gross ones). The sterility of the fruits can be, however, easily determined by counting the missing embryos in the linear array. A considerable number of these defects, perhaps most, are presumably due to large deficiencies and two- or multiple-hit aberrations of the chromosomes.

The mutation frequencies in Arabidopsis can easily be expressed on the genome basis, and the test results can be compared with those of any prokaryote or eukaryote. Mutagenicity information in Arabidopsis correlates very well with the carcinogenicty data for animal test systems. There is direct evidence (Rédei et al., 1980) that the metabolic system of of Arabidopsis can activate several promutagens into genetically effective compounds.

A mutagen assay with Arabidopsis can be completed within four to five weeks. Because of the small size of the plants, up to 200 or more individuals can be raised per 10-cm-diameter petri plate in inexpensive growth chambers. Therefore, the assays are not only fast and revealing, but they are also very inexpensive. The plants can be grown year round, both in the laboratory and in the environment (this species is winter-hardy even in the North). Though sufficient information is lacking on its utility for in situ testing of environmental pollutants, there is no apparent reason why it could not be employed successfully for this purpose too.

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SOYBEAN SYSTEM FOR TESTING THE GENETIC EFFECTS OF INDUSTRIAL EMISSIONS AND LIQUID EFFLUENTS

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INTRODUCTION

The testing of complex mixtures found in today's air, water, and soils for harmful effects on man is a gigantic and important task. This paper introduces a eukaryotic test system that yields at least qualitative estimates of genetic damage of the type observable in man. The soybean spot test is based on inducing various types of genetic damage by treating seeds or seedlings with the chemical or mixture of chemicals in question. This review briefly describes the nature of the test system, the kinds of data obtained for various chemicals, their postulated genetic effects, and the potential usefulness of this material for environmental mutagen testing. This summary is only a guideline to understanding the system; the details of previous work can be found in references (for reviews, see Nilan and Vig, 1976; Vig, 1975, 1978).

THE TEST SYSTEM

The Origin of Spots

In 1956, Weber and Weiss described a light-green colored plant of soybean (Glycine max [L.] Merrill) whose progency segregated in a ratio of 1 dark green: 2 light green: 1 golden yellow. The yellow color is due to lack of chlorophyll. The alleles controlling this trait are symbolized by Y_{11} and y_{11}: $Y_{11}Y_{11}$ plants are dark green, $Y_{11}y_{11}$ plants are light green, and $y_{11}y_{11}$ plants are golden yellow. In heterozygotes, the embryonic leaves (which develop into two simple, opposite leaves) and the first compound leaf occasionally show a few dark green spots and about an

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equal number of yellow spots. Although these spots resemble the leaves of the two homozygotes in color, the intensity of their colors depends on the number of layers of palisade cells involved in the mutational event. An altered embryonic leaf cell usually develops its colony of cells during division and expansion without being outcompeted by the surrounding tissue. The yellow spots survive because the light green background tissue provides the necessary carbohydrates for their growth.

Besides these single spots, the $Y_{11}y_{11}$ leaves also produce twin spots composed of adjacent equal-sized, mirror-image spots, one dark green and one yellow. The cells of such twin spots appear to have complementary genotypes, i.e., $Y_{11}Y_{11}$ and $y_{11}y_{11}$.

The origin of twin spots is best attributed to somatic crossing over in $Y_{11}y_{11}$ cells. The failure of one of these cells to develop into a visible sector results in a single dark green or yellow spot. The frequency of occurrence of these twin spots coincides with the frequency of somatic crossing over in several other organisms (see Vig, 1978) and can be dramatically increased by applying substances known to cause mitotic recombination, such as mitomycin C (German and LaRock, 1969; Holiday, 1954; Vig and Paddock, 1968).

The single yellow spots may also develop by the multiplication of a cell (or cells) that has lost the chromosome segment carrying the Y_{11} allele. Duplication of Y_{11} allele or deletion of the y_{11} allele, followed by cell multiplication, may give single dark green spots (Vig, 1969b). These situations may result from nonhomologous translocations involving the chromosome carrying this gene. Consequently, $Y_{11}y_{11}$ leaves treated with a given mutagen or complex mixtures of mutagens may be analyzed to distinguish between the modes of action of various mutagens. Furthermore, specific locus mutations may be induced (y_{11} to Y_{11}, in $y_{11}y_{11}$ cells) to give light green spots on a yellow background.

The Protocol

A 10- to 15-g sample of seed of variety T219, L65-1237, or L72-1937 is treated for the desired length of time with a solution of the chemical or mixture of chemicals to be tested. The seed is then thoroughly washed in running tap water, sown 6 mm (0.25 in.) deep in washed, coarse sand of no nutritive value in galvanized metal flats, and watered as required, depending on the temperature and humidity of the greenhouse. The protocol can be altered so that the seed is watered with a solution of the suspected mutagen. Agents like mitomycin C, caffeine, or nitrosourea may be used as positive controls.

The plants are ready for analysis in four to five weeks, when the second compound leaf unfolds. For both heterozygous and homogyzous plants, spots are counted on the two simple leaves and the first compound leaf, which is considered equivalent in area to three simple leaves. Usually, the simple leaves have more spots, due to a larger initial number of cells than in the compound leaf. Because the spontaneous background frequency of the control varies with the age of the seed, a statistical procedure such as the t-test is used to confirm the induction of damage by the mutagen. The doses and number of replicates required are determined by the chemical's mutagenic effectiveness.

STUDIES WITH SOME TEST AGENTS

The Induction of Twin Spots

Chemicals whose primary genetic effect is to cause somatic recombination produce a preponderance of twin spots on the heterozygous leaves. Two such chemicals, caffeine and mitomycin C, deserve special mention. Four-hour treatments with caffeine in concentrations ranging from 0.0625% to 0.5% increase the frequencies of all types of spots, especially doubles (Vig, 1973b). In one experiment, the ratio of total spots to twin spots was 11:1 in the control; for seeds treated for 4 h with 0.0625%, 0.125%, 0.25%, and 0.5% caffeine, the ratios were 3.6, 4.0, 3.4, and 2.7, respectively. Several other experiments have confirmed that about one third of the spots induced by caffeine are twin spots.

When seeds are treated with mitomycin C at a wide range of concentrations and at various stages of seed germination, usually one third or more of the spots are twins. The same frequency of twin spots occurs following treatment of seeds with mitomycin C solution and following application of the chemical in lanolin paste to the growing tip of the seedling, including the unexpanded third through fifth compound leaves (Vig and Paddock, 1968). In these studies, concentrations of mitomycin C were as low as 0.00325% (for 24 h) for seed treatments and 0.005% in lanolin paste. Twin spots are also preferentially induced by applying the chemical to the seed at various physiological ages for 4-h periods (i.e., from 0 to 4, 4 to 8, 8 to 12, 12 to 16,..., 32 to 36 hours after germination [Vig, 1973a]).

The alkylating agents diepoxybutane, trenimon, and methylmethane sulfonate affect the frequency of spots similarly. These three agents are potent inducers of spots of all types, about one third of which are twin spots. In the case of diepoxybutane, concentrations as low as 0.5 ppm applied to seed for 24 h are effective, and concentrations of trenimon as low as 0.25 ppm applied under similar conditions increase the total spot frequency to about twice that of the control (Vig and Zimmermann, 1977). Methylmethane sulfonate induces similar types of spots when applied at various phases of germination (Vig et al., 1976) and at concentrations as low as 6 ppm. At high concentrations, however, these alkylating agents affect leaf expansion drastically, resulting in a reduced spot frequency calculated on per leaf basis.

Preponderant Production of Yellow Spots

While ethyl methanesulfonate slightly increases the frequency of twin spots over that of the control, it is far more effective in producing yellow spots than the other two types of spots. In one study (Vig et al., 1976), treatment with a 0.25% solution of this chemical produced about 50% more single yellow spots than single dark green spots and about 2.5 times as many single yellow spots as twin spots. Although ethylmethane sulfonate and methylmethane sulfonate are closely related chemicals, they produce different frequencies of the various spot types at any given concentration (Vig et al., 1976).

Treatment of the seed with cobalt-60-emitted gamma rays gives results similar to those for ethylmethane sulfonate, although the relative frequency of yellow spots is even higher. This has been found over a range of 10 to 750 R for both dry and pre-soaked seed (Vig, 1974); the total frequency of spots is generally higher in leaves from pre-soaked seed. Exposure of seed to beta particles from tritiated water results in equal frequencies of the three types of spots (Vig, 1974; Vig and McFarlane, 1975). Even tritium concentrations as low as 0.01 μC/ml for 96 h (equivalent to 4.5 R) are effective in producing spots. These differences could be due to internal availability of beta particles to the DNA from within the organic fraction of the embryo (Vig and McFarlane, 1975).

A Lack of Production of Twin Spots

Treatment of seeds with sodium azide (NaN_3) greatly increases the frequencies of single dark green and yellow spots (Vig and McFarlane, 1975). However, the frequency of twin spots is barely above that of the control (Vig, 1973c), and the chemical generally does not produce light green spots on yellow leaves. Thus, these spots are probably not due to somatic crossing over, point mutations, or deletions of chromosome segments. We tentatively conclude that NaN_3 causes nondisjunction, giving $Y_{11}Y_{11}y_{11}$ sectors that are dark green and $Y_{11}y_{11}y_{11}$ sectors that are nearly yellow. The monosomic cell lines are presumably inviable or outcompeted by the normal and trisomic lines, and are thus lost. We have not found similar results for any other mutagen that we have tested.

The Induction of Point Mutations from y_{11} to Y_{11}

Light green spots on yellow ($y_{11}y_{11}$) leaves are produced by specific locus mutations, y_{11} to Y_{11}. Not all chemicals that induce spots in the heterozygotes also produce light green spots in yellow homozygotes. Caffeine produces such spots at concentrations of 0.05% or higher (Vig, 1973b). This effect distinguishes caffeine from chemicals like mitomycin C or nitrosoamines, which induce both twin spots and single spots on $Y_{11}y_{11}$ leaves, but have no effect on $y_{11}y_{11}$ leaves.

The alkylating agents methylmethane sulfonate, ethylmethane sulfonate, methylethane sulfonate, and methylbutane sulfonate cause mutation of y_{11} to Y_{11} at concentrations as low as 0.02% when applied to seed for 20 h (Vig et al., 1976). Diepoxybutane and trenimon also induce light green spots on yellow leaves. For these chemicals, a concentration of 0.25 ppm applied to seed for 24 h is mutagenic (Vig and Zimmermann, 1977); thus, these chemicals induce not only somatic recombination and chromosome deletions (as indicated by the induction of spots on $Y_{11}y_{11}$ leaves), but also point mutations of the allele y_{11} to Y_{11}.

No other chemical has been found to induce the mutation of y_{11} to Y_{11}. However, as in other such test systems, beta particles and gamma rays induce this point mutation at about the same frequency as they do spots on $Y_{11}y_{11}$ leaves (Vig, 1974, 1978; Vig and McFarlane, 1975).

MUTAGENS REQUIRING METABOLIC ACTIVATION

In recent years, increasing attention has been focussed on the mutagenic action of chemicals requiring metabolic activation. The S-9 fraction of rat liver homogenate is commonly used to activate promutagens. Recent studies indicate that liver is not the only system with the enzymatic machinery needed for such activation. In 1968, Veleminsky and Gichner demonstrated the mutagenic activity of some promutagens in plant systems without mammalian metabolic activation (see Arenaz and Vig, 1978; Klekowski and Levin, 1979).

We have treated soybean seeds with aqueous solutions of dimethylnitrosoamine at concentrations as low as 1.25 ppm for 24 h (Arenaz and Vig, 1978). At this dose, we found a 2.8-fold increase in the frequency of twin spots, a 2.6-fold increase in dark green spots, and 1.7-fold increase in yellow spots, demonstrating that this plant system can activate this chemical. Treatments with concentrations from 60 to 500 ppm appeared to cause maximal conversion of the chemical into true mutagen. Methylnitrosourea, a related nitrosoamide that does not require metabolic activation, showed no such saturation effect. However, methylnitrosourea is much more toxic, in that interference with leaf expansion (yielding an artificially low spot frequency) occurs at 125 ppm. In comparison, dimethylnitrosoamine is tolerated by the plant at doses as high as 500 ppm (Arenaz and Vig, 1974).

Despite data demonstrating metabolic activation in the plant, it remains to be shown that metabolites produced through the mediation of plant extracts (e.g., from nitrosoamines, atrazine, and other such chemicals) can cause mutations or chromosome changes in any mammalian system.

CONCLUSIONS

The soybean spot test is well suited for assaying both pure chemicals and complex mixtures. The results for several agents tested in this system are summarized in Tables 1 and 2. The system allows not only quick and inexpensive assessment of the genetic damage in a eukaryotic system, but also discrimination among different genetic end points and thus among modes of action of the agents. Thus, a chemical like methylmethane sulfonate, which can cause DNA crosslinks, induces twin spots and single spots on $Y_{11}y_{11}$ leaves and light green spots on $y_{11}y_{11}$ leaves, unlike NaN_3, which produces only single spots on $Y_{11}y_{11}$ leaves and apparently has no other effect. This discriminatory ability of the system is advantageous in preliminary screening.

The soybean test system is suitable for use with complex mixtures. Klekowsky and Levin (1979) recently showed that effluent from paper mills induces spots on $Y_{11}y_{11}$ leaves. The system should be adaptable to testing of liquid, solid, or gaseous effluents, as long as the seed or the seedlings (as in Vig and Paddock, 1968) can be treated at appropriate stages of development.

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Table 1. Summary of Qualitative Genetic Effects of Agents Tested for Induction of Spots on the Leaves of the Soybean (Glycine max)

Agent	$Y_{11}y_{11}$ Plants		$y_{11}y_{11}$ Plants	Postulated Primary Mechanism	Reference
	Twin Spots	Single Spots	Single Spots		
Mitomycin C	+	+	–	a,b	Vig and Paddock, 1968
Caffeine	+	+	+	a,b,c,	Vig, 1973b
Actinomycin	+	+	–	a,b	Vig, 1973b
Daunomycin	–	±	–	b	Vig and Paddock, 1968
5-Fluorodeoxy uridine	–	+	–	b	Vig, 1973b
N-methyl, N-nitro, N-nitrosoguanidine	+	+	–	b	Arenaz and Vig, 1976
Ethylmethane sulfonate	±	+	+	b,c	Vig et al., 1976
Methylethane sulfonate	(not tested)		+	c	Vig et al., 1976
Methylmethane sulfonate	+	+	+	a,b,c	Vig et al., 1976
Methylbutane sulfonate	(not tested)		+	c	Vig et al., 1976
Colchicine	+	+	–	a	Ashley, 1978; Vig, 1969b
Puromycin	–	±	–	b	Vig, 1973b
Sodium azide	±	+	–	d	Vig, 1973c
N-methyl-N-nitrosurea	+	++	–	b	Arenaz and Vig, 1978
Dimethyl nitrosamine	+	++	–	b	Arenaz and Vig, 1978
Trenimone	+	+	+	a,c	Vig and Zimmermann, 1977
Diepoxybutane	+	+	+	a,c	Vig and Zimmermann, 1977
Carofur (1-[5-nitro-2-furyl]-2-[6-amino-3-pyridazyl]-ethanehydrochloride)	+	+	–	a	Vig and Zimmermann, 1977

[a] Somatic crossing over usually with accompanying chromosome breakage.

[b] Chromosome breakage as primary cause.

[c] Point mutation.

[d] Nondisjunction (?).

Table 2. Agents That Did Not Induce Spots on the Leaves of the Soybean (*Glycine max*)

Agent	Reference
Aminoazo toluene	Arenaz, 1978
Nucleosides, d-A d-C d-G d-T	Vig, 1972
Aluminum potassium nitrate	Vig and Mandeville, 1972
Copper sulfate	Vig and Mandeville, 1972
Ferrous sulfate	Vig and Mandeville, 1972
Hycanthone	Arenaz, 1977
Ammonium thiocyanide	Vig, 1975
1-nitroso-2-naphthyl-2, 6-disulfonic acid	Vig, 1975
Cytosine arabinoside	Vig, 1975
2-Chloroethanol	Vig, 1975
Hydroxylamine hydrochloride	Vig, 1975
Urea	Vig, 1975
N, N-dinitrosopiperazine	Vig, 1975
N-nitroso-methyl urea	Vig, 1975

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MUTAGENICITY OF NITROGEN COMPOUNDS FROM SYNTHETIC CRUDE OILS: COLLECTION, SEPARATION, AND BIOLOGICAL TESTING

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INTRODUCTION

Short-term mutagenesis assays have been used to test complex environmental mixtures, in order to 1) serve as predictors of long-range health effects, 2) guide chemical separation procedures for the isolation and concentration of biologically active materials, 3) identify chemical agents responsible for biological activity, and 4) determine priorities for further, extensive testing. Organic extraction coupled with chemical-class fractionation is a prerequisite for most of these assays.

Our emphasis has been on evaluating various test materials from the newly emerging synfuel technologies. We have previously used the class chemical separation procedure developed by Swain et al. (1969) to fractionate certain coal-derived (Epler et al., 1978) and shale-derived (Epler et al., 1979b) oils for mutagenicity testing. To minimize chemical reactivity during the fractionation procedure, several synfuels were separated with Sephadex LH-20 gel chromatography (Rao et al., in press). The bacterial mutagenicity assay developed by Ames (Ames et al., 1975) employs certain well-characterized histidine-auxotrophic mutants of Salmonella typhimurium. Mutagenicity results indicate that the alkaline fractions containing azaarenes, primary amines, and nitro polyaromatics are mutagenic, along with the neutral-fraction-containing polycyclic aromatic hydrocarbons (PAH) and nitrogen-containing PAH (Ho et al., in press). The biological activity of these organic compounds is characterized by the ability to revert the frameshift strains TA1538 and TA98 (Rao et al., 1978) and dependence on specific activation systems.

Source of Samples

Samples used in this study were supplied by U.S. Environmental Protection Agency/U.S. Department of Energy Synfuel Research Materials Facility (Coffin et al., 1979). Process operating conditions at the time of sampling, sampling conditions, and sample histories are not sufficiently defined to allow process-specific conclusions. Samples (with repository numbers) and their sources are listed in Table 1. A detailed process description has been presented elsewhere (Guerin et al., in press).

Table 1. Synfuel Samples and Their Sources

Sample[a]	Source
Petroleum	
Wilmington crude oil (5301)	Bartlesville Energy Technology Center
Recluse crude oil (5305)	Bartlesville Energy Technology Center
Shale-derived oils	
Shale oil (*in situ*) (4101)	Laramie Energy Technology Center
Paraho shale oil (4601)	US Navy/Standard Oil Co. of Ohio
HDT-Paraho shale oil (4602)	US Navy/Standard Oil Co. of Ohio
Coal-derived oils	
SRC II fuel oil (1701)	Pittsburgh and Midway Mining Co.
H-coal dist.--raw (1601)	Mobil Research/EPRI
H-coal dist.--HDT low severity (1602)	Mobil Research/EPRI
H-coal dist.--HDT medium severity (1603)	Mobil Research/EPRI
H-coal dist.--HDT high severity (1604)	Mobil Research/EPRI
$ZnCl_2$ dist. (1801)	Conoco Coal Development Co.

[a] Respository numbers in parentheses.

Purpose

The objective of this study was to identify mutagenic activity in fractionated synfuel samples and to isolate and identify the mutagenic chemical agents.

MATERIALS AND METHODS

Chemical Fractionation

The chemical fractionation procedure (Figure 1) has been described by Guerin et al. (in press). Acidic and basic fractions were separated by liquid/liquid partitioning into ether-soluble and -insoluble acids and bases (water-soluble fractions were generally inactive in the mutagenicity assays). The neutral fraction was separated using Sephadex LH-20 into aliphatic, aromatic, polyaromatic, and polar fractions by isopropanol elution. Solvents were removed by rotary evaporation; the residue was dissolved in dimethylsulfoxide for mutagenicity assays. The basic fraction was subfractionated by use of basic alumina and Sephadex LH-20. The ether-soluble base fraction was loaded onto the column and eluted with 500 ml benzene (benzene subfraction) followed by 700 ml ethanol. Ethanol was removed by rotary evaporation, and the residue was separated further on a Sephadex LH-20 gel column. The column was eluted sequentially with 250 ml of isopropanol (isopropanol subfraction) and 600 ml of acetone (acetone subfraction), and the eluting solvent was removed by rotary evaporation.

Mutagenicity Assay

Histidine-auxotrophic strain TA98 of *S. typhimurium*, obtained through the courtesy of Dr. B.N. Ames (University of California at Berkeley), was used for these studies. The procedure (based on the work of Ames et al., 1975) was to overlay minimal-medium agar plates with soft agar containing the fraction being tested, bacterial cells (2×10^8), and liver homogenate (S-9 mix) from Aroclor-1254-induced rats (for metabolic activation). Activity in revertants per milligram of test substance was derived from the slope of the induction curve. Total mutagenic activity of a starting material was computed from the activities of its subfractions corrected for the percentages by weight contributed by the subfractions to the starting material.

RESULTS AND DISCUSSION

The crude oils could not be tested for mutagenicity because of their toxicity; when tested, they yielded questionable results. To overcome this problem and also to obtain a more homogeneous distribution of sample in the test medium, the samples were fractionated by the general procedure described above (see Figure 1). Recovery and reproducibility have been tested by using triplicate samples of shale oil and crude oil. Chemical recovery

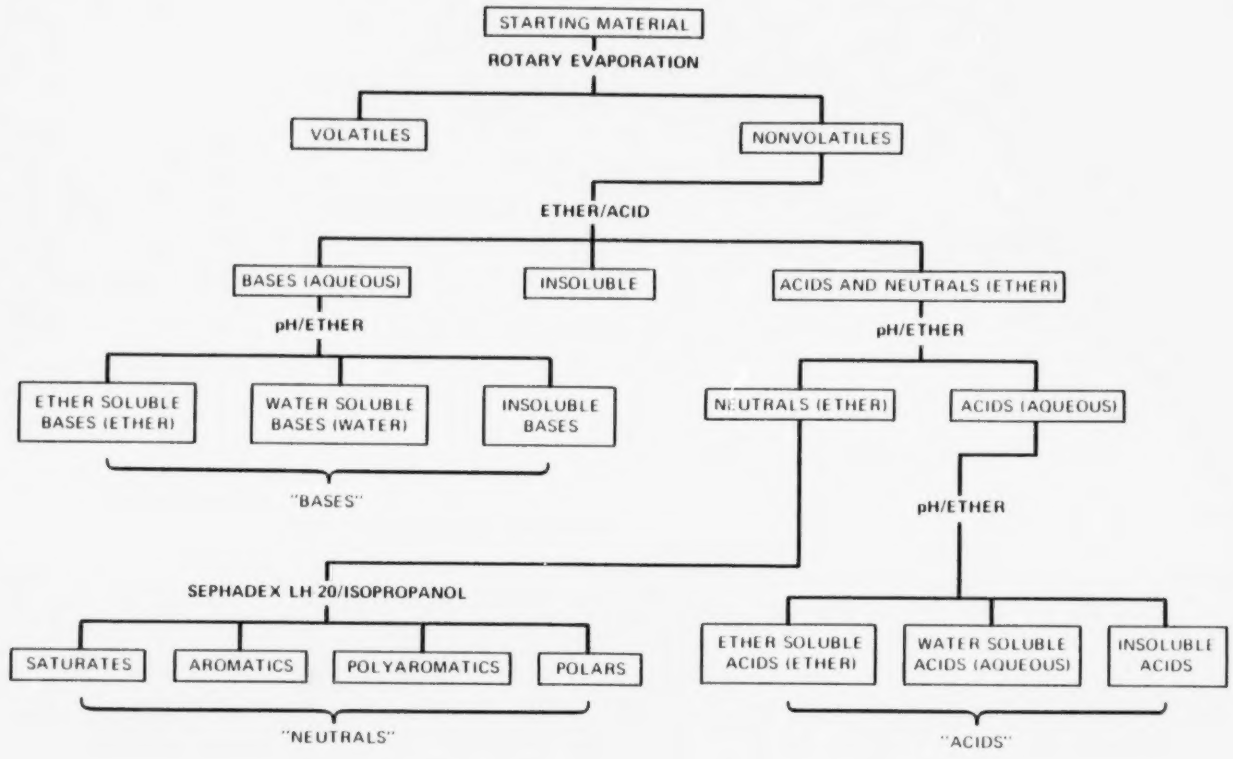
STARTING MATERIAL
ROTARY EVAPORATION
VOLATILES
NONVOLATILES
ETHER/ACID
BASES (AQUEOUS)
INSOLUBLE
ACIDS AND NEUTRALS (ETHER)
pH/ETHER
pH/ETHER
ETHER SOLUBLE BASES (ETHER)
WATER SOLUBLE BASES (WATER)
INSOLUBLE BASES
NEUTRALS (ETHER)
ACIDS (AQUEOUS)
"BASES"
pH/ETHER
SEPHADEX LH 20/ISOPROPANOL
SATURATES
AROMATICS
POLYAROMATICS
POLARS
ETHER SOLUBLE ACIDS (ETHER)
WATER SOLUBLE ACIDS (AQUEOUS)
INSOLUBLE ACIDS
"NEUTRALS"
"ACIDS"

Figure 1. Chemical fractionation procedure (from Guerin et al., in press). Materials are shown in boxes, their phases are indicated in parentheses, and the steps of the procedure are given in boldface type.

and reproducibility are generally adequate for biological testing purposes, even though recoveries are unacceptably low with certain samples. The losses are caused by volatile matter, which is difficult to use in bioassays.

Table 2 gives the distribution of mutagenic activity in petroleum oils, shale oils, and coal-derived oils. The acidic fractions were inactive in the mutagenicity assays. All of the activity was found in the basic and neutral fractions: the activity levels varied from sample to sample. The petroleum samples showed activity in the neutral fraction, which was weakly mutagenic. In the shale oil samples, high in nitrogen, both the neutral and basic fractions were mutagenic. Significant mutagenic activity was also observed in both the neutral and basic fractions of the coal-derived oils. The shale oils and coal-derived oils were more mutagenic than were the petroleum samples.

Hydrotreatment (HDT) seemed to reduce mutagenic activity, as seen in the results obtained with HDT-hydrogenated coal (H-Coal) distillates. The high severity hydrotreatment completely eliminated mutagenic activity from the H-Coal sample, while the medium and low severity treatments were less effective. Zinc chloride- ($ZnCl_2$) catalyzed distillation apparently eliminated alkaline mutagens from the coal-derived oil.

Mutagenic activity in the basic and neutral fractions led us to examine these fractions to isolate and identify chemical agents responsible for the biological activity. When the basic fraction was subfractionated, biological activity was found in the ultimate acetone fraction that comprised approximately 10% of the starting ether-soluble basic (ESB) fraction. Results for subfractionated SRC-II ESB and shale oil ESB are given in Table 3. The acetone fractions with high specific activities can be used for biological assays in other genetic systems as well as for chemical analysis (Epler et al., 1979a). Azaarenes and primary aromatic amines are the organic constituents of this fraction suspected of causing the mutagenic activity.

Chemical separation and analysis of the neutral fraction suggested PAH, alkylated PAH (Griest et al., 1979), and nitrogen-PAH (Ho et al., in press) as the possible mutagenic agents. Mutagenic activity of PAH and alkylated PAH appeared to increase with the ring size (see Figure 2). The aliphatic constituents of coal-derived materials were not mutagenic, while the nitrogen-PAH fraction obtained from coal oil (Ho et al., in press) had a specific activity more than twice that of the PAH fraction (Table 4). The nitrogen-PAH fraction from a shale-derived oil was not mutagenic.

Table 2. Distribution of Mutagenic Activity in Synfuels[a]

Sample	Total Mutagenic Activity (revertants/mg)[b]	Distribution of Activity (%)			
		Neutral	Acids	Bases	Other
Petroleum					
Wilmington crude oil	5	100	0	0	0
Recluse crude oil	6	100	0	0	0
Shale-derived oils					
Shale oil (*in situ*)	178	54	2	42	2
Paraho shale oil	390	31	0	69	0
HDT-Paraho shale oil	0	0	0	0	0
Coal-derived oils					
SRC-II fuel oil	1000	65	0	35	0
H-coal dist.--raw	350	63	0	37	0
H-coal dist.--HDT low severity	540	100	0	0	0
H-coal dist.--HDT medium severity	210	–	–	–	–
H-coal dist.--HDT high severity	0	0	0	0	0
$ZnCl_2$ dist.	530	100	0	0	0

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[a] From Guerin et al. (1980, in press).

[b] Determined from the linear portion of a dose-response curve with strain TA98.

Table 3. Distribution of Mutagenic Activity in the Ether-soluble Basic Fraction (ESB)

Test Substance	Relative Weight (%)	Specific Activity (rev/mg)	Weighted Activity (rev/mg)
SRC-II ESB	-	0	14,000
Benzene	74	0	0
Isopropanol	6	400	24
Acetone	15	68,000	10,200
Total	95		10,224
Shale oil ESB	-	-	2,500
Benzene	77	0	0
Isopropanol	12	0	0
Acetone	9	20,000	1,800
Total	98		1,800

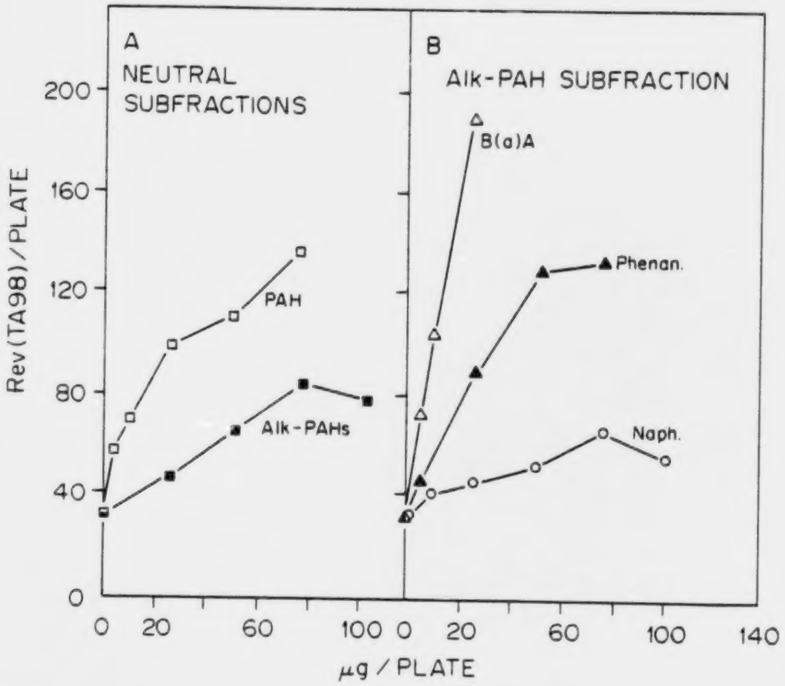

Figure 2. Mutagenicity of the polycyclic aromatic hydrocarbon (PAH) and alkylated (PAH) subfractions, including benz(a)anthracene, phenanthrene, and naphthalene.

Table 4. Mutagenic Activities of Neutral Subfractions of a Coal-derived Oil and a Shale Oil[a]

Subfraction	Specific Activity (rev/mg)	
	Coal-derived Oil	Shale Oil
Aliphatic (AL)	0	0
PAH (I)	1390	120
Neutral N-PAH (II)	3250	0
Polar (III)	3380	1100

[a] From Ho et al. (in press).

CONCLUSIONS

Our conclusions are as follows: 1) Short-term bioassays such as the Ames *Salmonella* histidine reversion assay can be effectively applied to complex environmental samples. 2) Proper chemical extraction and fractionation methods should be coupled to the assay. 3) The shale- and coal-derived oils were relatively more mutagenic than was petroleum crude oil. 4) Mutagenic activity was mainly associated with the basic and neutral fractions. 5) Azaarenes, aromatic amines, PAH, alkylated PAH, and nitrogen-PAH were the organic constituents of these fractions suspected of causing the mutagenic activity.

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THE DETECTION OF POTENTIAL GENETIC HAZARDS USING PLANT CYTOGENETICS AND MICROBIAL MUTAGENESIS ASSAYS

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INTRODUCTION

The recent realization that industrial effluents and wastes could pose health hazards to future generations has led to considerable research on the health effects of these substances and to efforts to regulate their release. The Research Conservation and Recovery Act (RCRA) specifically addresses the potential health and environmental hazards of solid wastes. Evaluation of long-term consequences of exposure to the huge number of potentially hazardous compounds and complex mixtures would tax the scientific community's whole-animal testing capabilities. Therefore, many short-term bioassays have been developed to rapidly screen compounds for toxicity, mutagenicity, teratogenicity, and carcinogenicity. It is hoped that these short-term tests will predict health hazards early in the development of new technologies, allowing technological changes to be made at early stages, and resulting in timely whole-animal testing where screening indicates potential hazards. Time and money should thus be saved in both technological development and biological testing.

In the approach that we have taken with an array of complex mixtures from the technological world, chemical characterization and preparation are coupled with short-term bioassays. Other such investigations have usually involved chemical analyses and preparation for biological testing (e.g., cytotoxicity, mutagenicity, and carcinogenicity assays). The genetic test for *Salmonella* histidine-mutant reversion (Ames et al., 1975) has been widely used to screen potential mutagens or carcinogens. Subsequent fractionation procedures are carried out to isolate and identify the mutagens in the material; the bioassay is used to trace the biological activity and guide the separations. The biological tests function as 1) predictors of long-range health effects such as mutagenesis, teratogenesis, or carcinogenesis; 2) predictors of toxicity to man and his environment; 3) a mechanism to rapidly isolate and identify hazardous agents in complex material; and 4) indicators of relative biological activity, through the correlation of control data with changes in environmental or process conditions.

Plant systems may offer an additional means of evaluating complex mixtures. According to Kihlman (1971), root tips are the ideal plant tissue in which to study the effects of chemicals on chromosomes because 1) they are readily available throughout the year; 2) they are inexpensive; 3) they are easy to handle; 4) they provide data within a few days; 5) they are directly exposed to the chemical in aqueous solutions of known concentrations; 6) they provide numerous dividing cells for analysis; and 7) they have few, relatively large chromosomes.

Barley (Hordeum vulgare [L] emend Lam.) has 2n = 14 chromosomes, ranging from 6 to 8 μm in length. The barley embryo has from five to seven seminal roots that yield numerous dividing cells for analysis within 24 to 48 h after germination starts. Following seed treatments, the effects of chemical and physical agents can be assessed in terms of cytogenetic and genetic end points in the same population of plants. This unique advantage has made barley a useful mutagenesis test organism.

The most frequently used method for studying the effects of chemicals on the chromosomes of barley root tip cells is the analysis of anaphases for detectable aberrations (Nicoloff and Gecheff, 1976). The assay has been used to test chemicals either suspected or known to be mutagens; generally, the results have agreed with those from other organisms tested with the same compounds. The assay has not been used widely to test complex mixtures.

In this report, we present data from a study comparing microbial mutagenicity assays (Salmonella and yeast) with a plant cytogenetic assay. The materials used were aqueous extracts from a fly ash sample and an arsenic- (As) contaminated groundwater sample (provided by the U.S. Environmental Protection Agency). The purpose of this research was to assess the potential of complex environmental mixtures (extracts of solid wastes) to produce cytogenetic effects in a higher plant and gene mutations in microbes. A complex environmental mixture capable of inducing both end points warrants close scrutiny as a potential health hazard to humans.

MATERIALS AND METHODS

Preparation of Liver Homogenate (S-9)

The microsomal preparation was made according to Ames et al. (1975). The livers of Sprague-Dawley rats (induced with either Aroclor 1254 [Ar S-9] or phenobarbital [ψB S-9]) were washed in an equal volume of 0.15 M NaCl, minced with sterile scissors, and homogenized with a Potter-Elvehjem apparatus in 3 vol of 0.15 M KCl. The homogenate was centrifuged at 4°C for 10 min at 9000 x g. The supernatant was collected and stored at -80°C. The activation system (S-9) mix contained, per milliliter, 0.3 ml of liver homogenate, 8 μmol magnesium chloride, 33 μmol potassium chloride, 5 μmol glucose-6-phosphate, and 4 μmol nicotinamide adenine dinucleotide phosphate in 100 μmol of sodium phosphate buffer (pH 7.4).

Bacterial Mutagenicity Assay

Among bacterial mutagenicity test systems, the assay developed by Ames using the histidine auxotrophic strains of *Salmonella typhimurium* is widely used as a screening test to detect potential genetic and carcinogenic hazards. Of the four standard tester strains generally used in the assays, the missense strains TA100 and TA1535 detect base-pair substitutions, while strains TA1537 and TA98 detect frameshift mutations. The general procedure was described by Ames et al. (1975). A bacterial suspension (2 x 10^8/ml) was added to 2 ml of molten top agar (45°C) containing test substance. S-9 mix (0.5 ml/plate) was added, when required, to provide metabolic activation. The top agar was overlayed on Vogel-Bonner (1956) minimal medium, and revertant colonies were counted after two days of incubation at 37°C. Known chemical mutagens as positive controls and solvent (negative) controls were routinely run.

Yeast Assays

The *Saccharomyces* assay measures both forward and reverse mutation. Forward mutation is detected by the inactivation of the arginine permease gene (*CAN1*), leading to resistance (*can*^{r}) to the toxic antimetabolite canavanine. Reverse mutation is monitored by use of a histidine auxotroph (*his1-7*) that reverts by base-pair substitution.

The test strain used in these experiments was constructed from stocks of *Saccharomyces cerevisiae* maintained at Oak Ridge National Laboratory (ORNL) and from stocks obtained from the Berkeley collection. Strain XL7-10B has the genotype α p^+ CAN1 his1-7 lys1-1 ura1.

Details of the preparation of media, rat-liver homogenates, and the mutagenicity assay have been described by Larimer et al. (1978, 1980). The test material was mixed with yeast cells in buffer or rat-liver homogenate (S-9) in buffer and incubated for 3 or 24 h at 30°C with shaking before it was plated on selective media. Mutant clones were scored on selective plates after incubation for five days at 30°C. To determine survival, dilutions were plated on yeast-extract-peptone-dextrose plates and scored after two days at 30°C.

Plant Cytogenetic Assay

Seeds of Himalaya barley from R.A. Nilan (Washington State University, Pullman, WA) were stored under refrigeration and hand-picked for quality just prior to each experiment. Approximately 25 seeds were sown embryo-side-up on Whatman #1 filter paper in glass petri plates. The filter paper was thoroughly saturated by adding 7.5 ml of either double-distilled water or a test solution at pH 7.0. Each treatment was done in triplicate; plates were placed in sealed polyethylene bags and cultured for 42 to 46 h at 25°C under 10 to 15 $\mu E\ m^{-2} sec^{-1}$ fluorescent light.

Germinating seeds were killed and fixed in 3:1 ethanol-glacial acetic acid and stored in the refrigerator until the roots were processed. Excised roots were hydrolyzed for 9 min in 1 N HCl at 60°C, reacted with Schiff's Reagent for at least 15 min, treated with pectinase (0.4% in water, pH 4.0) for at least 30 min, and squashed in aceto-carmine; Deckglaskitt was used to seal around the coverslip. Cells were observed at 1000X magnification for the presence of bridges and fragments at anaphase.

Data were expressed as aberrations per hundred anaphases and as percentage of aberrant anaphases. Statistical inferences were drawn on the bases of analysis of variance and chi-square analysis of 2 x 2 contingency tables for aberrant vs. normal anaphases in control vs. chemical treatments.

Preparation of Samples

Aqueous extracts of the fly ash samples were prepared according to the extraction procedure (EP) given in the Federal Register (1978). The As-contaminated sample was used as provided by the U.S. Environmental Protection Agency. Resin concentration (XAD-2; Isolab, Inc.) procedures were described by Epler et al.

(1980). A 500-ml aqueous extract was passed through 4 g XAD-2 at a flow rate of 1.2 ml/min. The column was rinsed with deionized water and then eluted with acetone. The acetone eluate was evaporated to dryness, and the sample was dissolved in 2 ml dimethylsulfoxide.

RESULTS AND DISCUSSION

Salmonella Assay

Aqueous samples from the As-contaminated groundwater and its XAD-2 concentrate (12.5-fold) were tested for mutagenicity over a nontoxic dose range. The results are given in Figure 1. The groundwater sample was not mutagenic (Figure 1A), even with metabolic activation, for strains TA98 and TA100. However, XAD-2 concentrate of groundwater (Figure 1B) exhibited a clear dose response with the frameshift strains TA1537 and TA98 and the highly sensitive TA100 strain. The missense strain TA1535 was not reverted. These results suggest the presence of a mutagenic constituent(s) in the aqueous sample whose activity was evident only when the sample was concentrated by an appropriate method.

The EP extract and its XAD-2 concentrate from fly ash sample were not mutagenic when tested with the basic set of tester strains (see Table 1). Addition of the metabolic activation system did not influence the result. Lack of mutagenic activity of fly ash from power plants conflicts with earlier reports of Chrisp et al. (1978) and Kubitschek and Venta (1979), who used serum extraction, and Hobbs et al. (1979), who used dimethylsulfoxide for extraction. The EP extraction procedure is probably not adequate to extract mutagenic agents from fly ash. When benzene extraction was used with a similar fly ash sample from a different power plant, a dose-dependent increase in the induction of histidine revertants was observed (unpublished data). Results obtained with known chemical mutagens (positive controls) are given in Table 1B. The alkylating agents ethylmethane sulfonate (EMS) and methylmethane sulfonate (MMS) were very specific in reverting TA1535 and TA100, respectively. The frameshift strains were reverted by 8-aminoquinoline (8-AmQ), specific to TA1537, and benzo(a)pyrene (B[a]P), which is mutagenic only when activated with Aroclor-1254-induced rat-liver homogenate (Ar S-9 mix).

Saccharomyces Mutation Assay

The As-contaminated groundwater sample was not mutagenic (see Table 2). The XAD-2 concentrate of this sample was mutagenic without metabolic activation for a 24-h exposure, giving a dose-dependent response. Metabolic activation appeared to reduce the

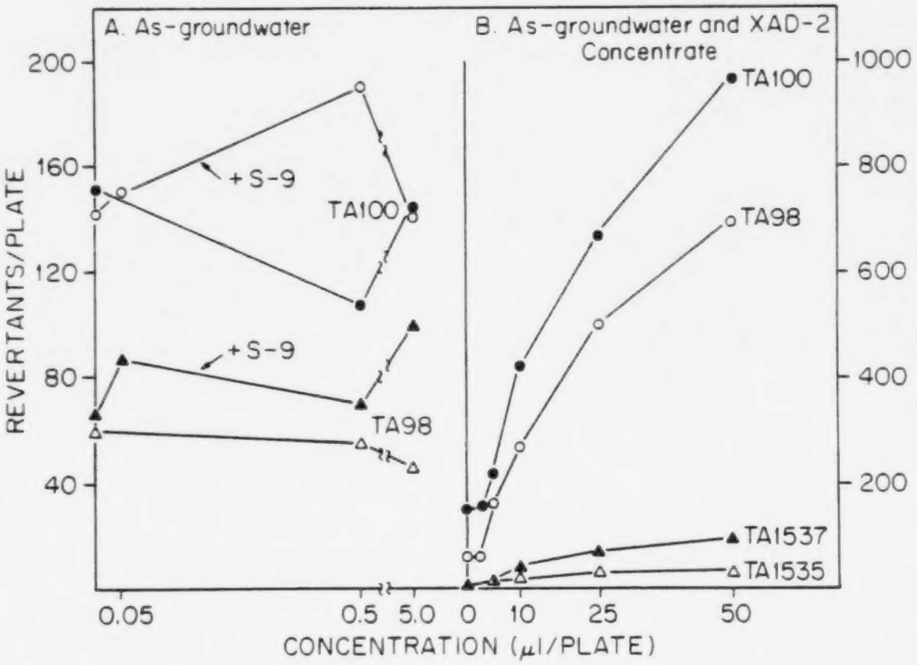

Figure 1. Mutagenicity of As-contaminated groundwater and its XAD-2 concentrate in the Salmonella assay. Each point represents an average from at least three independent experiments.

mutagenic potential of the XAD-2 concentrate. Neither of these test materials was toxic. More induced forward mutations to can^r were seen than hisl-7 base-pair substitutions. This is the typical response of this system to a frameshifting agent. The fly ash EP extract and its XAD-2 concentrate were not toxic or mutagenic to yeast (Table 3).

Plant Cytogenetic Assay

The cytogenetic effects observed in barley root tip cells are presented as percentage of aberrant anaphases in Table 4, which shows the distribution of anaphases into normal and aberrant classes for negative, positive, and solvent controls and complex environmental mixtures. The probability values were determined through chi-square tests for independence of the treatments and the proportions of aberrant anaphases, in 2 x 2 contingency tables.

Table 1. *Salmonella* Mutation Assay: Fly Ash--Aqueous Extract (EP) and its Concentrate (XAD-2)

Treatment and Concentration (μm/plate)	his^+ Revertants/Plate					
	TA1535[a]	TA1537[a]	TA98		TA100	
	EP	EP	EP	XAD-2	EP	XAD-2
No activation						
Solvent control	21	14	32	57	136	231
50	18	15	27	30	141	180
Ar S-9 activation						
Solvent control	15	16	46	33	192	209
10	15	14	35	42	108	203
25	8	15	34	28	141	206
50	16	15	53	26	123	206
75	16	18	42	38	172	208
Positive controls[b]						
50 μl (5%) EMS	704	11	48	-	386	-
50 μl (5%) MMS	42	13	48	-	1495	-
20 μg 8-AmQ	6	102	56	-	270	-
20 μg B(a)P + S-9	7	122	625	-	1700	-

[a] XAD-2 not tested with these strains.

[b] Underscoring represents positive response to mutagen.

The solvent controls (phosphate buffer and acetic acid extraction solution) yielded aberrant anaphases at frequencies not significantly different from those of the negative control, distilled water. In contrast, the frequency of aberrant anaphases was increased in the seeds treated with EMS, a positive control, and in those treated with the fly ash extract and the As-contaminated groundwater. The fly ash extract was used as we received it, except for the adjustment to pH 7.0, whereas the As-contaminated groundwater was diluted 1:8 and 1:16 with distilled water (1.25 and 0.625 ml extract diluted to 10 ml of solution), and these results were pooled. The more concentrated solutions were toxic to the germinating seed; i.e., germination was delayed and root growth was inhibited.

Table 2. Yeast Mutation Assay: Arsenic-Contaminated Groundwater and Its XAD-2 Concentrate

Concentration (µl)	% Survival				*can*^{r}/10^7 Survivors				*his*$^+$/10^7 Survivors			
	3 h		24 h		3 h		24 h		3 h		24 h	
	Sample	XAD-2	Sample	XAD-2	Sample	XAD-2	Sample	XAD-2	Sample	XAD-2	Sample	XAD-2
No activation												
Control	100	100	100	100	27	24	19	15	6	13	11	6
0.1	71	-	94	-	23	-	16	-	16	-	8	-
1.0	84	-	81	-	20	-	19	-	8	-	8	-
10	75	116	85	95	13	19	19	17	13	8	9	9
20	-	127	-	98	-	15	-	34	-	8	-	24
50	-	138	-	93	-	17	-	69	-	6	-	37
100	89	156	99	86	16	17	13	176	9	9	11	55
ΨB S-9 activation												
Control	100	100	100	100	14	17	17	15	12	10	12	8
0.1	107	-	103	-	21	-	14	-	10	-	9	-
1.0	59	-	87	-	27	-	21	-	10	-	10	-
10	82	105	103	94	21	18	14	19	14	11	9	12
20	-	98	-	86	-	20	-	31	-	13	-	6
50	-	103	-	79	-	16	-	89	-	8	-	29
100	91	112	119	71	18	21	18	107	12	7	7	24
Ar S-9 activation												
Control	100	100	100	100	17	15	21	14	9	9	11	6
0.1	97	-	94	-	18	-	20	-	11	-	9	-
1.0	92	-	96	-	13	-	27	-	8	-	7	-
10	87	107	91	108	21	12	22	13	12	10	10	8
20	-	111	-	122	-	25	-	41	-	11	-	9
50	-	122	-	91	-	13	-	74	-	5	-	17
100	88	90	103	82	24	13	22	118	11	8	13	38
EMS, 1% v/v	22				448				1270			

Table 3. Yeast Mutation Assay: Fly Ash EP Extract and its XAD-2 Concentrate

Concentration (µl)	% Survival				can^r/10^7 Survivors				his+/10^7 Survivors			
	3 h		24 h		3 h		24 h		3 h		24 h	
	EP	XAD-2	EP	XAD-2	EP	XAD-2	EP	XAD-2	EP	XAD-2	EP	XAD-2
No activation												
Control	100	100	100	100	12	26	19	24	13	10	13	6
0.1	104	-	101	-	18	-	18	-	5	-	10	-
1.0	109	-	96	-	14	-	13	-	6	-	15	-
10	94	103	97	96	14	25	12	27	9	6	16	8
20	-	105	-	98	-	27	-	25	-	6	-	7
50	-	102	-	86	-	23	-	22	-	8	-	8
100	97	96	99	92	11	23	16	23	9	9	10	10
PB S-9 activation												
Control	100	-	100	100	17	24	16	23	11	9	9	8
0.1	106	-	107	-	20	-	11	-	9	-	8	-
1.0	100	-	102	-	12	-	13	-	3	-	14	-
10	101	-	98	102	17	25	17	19	9	6	6	9
20	-	-	-	107	-	23	-	19	-	8	-	11
50	-	-	-	96	-	18	-	22	-	11	-	7
100	94	-	109	89	10	29	13	27	6	9	8	11
Ar S-9 activation												
Control	100	100	100	100	21	22	14	25	13	6	10	11
0.1	95	-	93	-	17	-	11	-	16	-	8	-
1.0	93	-	103	-	13	-	10	-	8	-	14	-
10	103	90	92	102	13	20	19	19	12	9	5	9
20	-	91	-	94	-	21	-	20	-	8	-	12
50	-	86	-	92	-	28	-	25	-	8	-	7
100	109	80	97	84	14	23	13	18	11	6	7	10
B(a)P, 100 µg Ar-activation			89				181				52	

Table 4. Chromosome Aberrations in Barley Embryos Treated with Extracts of Complex Environmental Mixtures: Numbers of Aberrant Anaphases

Treatments	Normal Anaphases N	Aberrant Anaphases N	Aberrant Anaphases %	P[a]
Distilled water	2182	48	2.15	
Phosphate buffer[b]	2197	75	3.30	0.05 to 0.10
EMS[c]	2241	184	7.59	< 0.001
Acetic acid extraction solution[d]	3784	88	2.27	0.30 to 0.50
Fly ash extract	2874	437	13.20	< 0.001
As-contaminated groundwater	1221	190	13.47	< 0.001

[a] P = probability that difference was due to chance, according to chi-square test for independence.

[b] Monobasic and dibasic phosphate buffer at pH 7.0.

[c] EMS at 0.025 M in phosphate buffer at pH 7.0; seeds soaked aerobically in water at ~ 1°C for 16 h and in EMS for 2 h at ~ 1°C plus 6 h at 24°C, rinsed, and cultured on a water-saturated Whatman #1 filter.

[d] Weak acetic acid extraction solution as described in Federal Register (1978).

Table 5 shows the same experimental data as in Table 4, but expressed as aberrations per hundred cells (the mean for each treatment). An arcsine transformation of the data (arcsin p, where p is a proportion) was done prior to the analysis of variance, to reduce the heterogeneity of the treatment variances. A Student-Newman-Keuls comparison of treatment means (Steel and Torrie, 1960) showed no difference between the negative control (distilled water) and either of the solvent controls (phosphate buffer and acetic acid extraction solution), whereas the positive control (EMS) and the two complex environmental mixtures (fly ash extract and the As-contaminated groundwater) induced a significantly greater number of aberrations per hundred anaphases.

Although the two methods of data analysis address different aspects of the cell population's response to seed treatment, the conclusion is the same: the barley root tip system responded to an unknown mutagenic substance(s) in the two complex environmental

Table 5. Chromosome Aberrations in Barley Embryos Treated with Extracts of Complex Environmental Mixtures: Aberrations per Hundred Cells

Treatments[a]	Embryos	Mean Number of Aberrations/ 100 Cells[b]	Standard Deviation
Distilled water	60	2.75	3.04
Acetic acid extraction solution	60	2.57	2.85
Fly ash extract	60	16.30	9.10
Phosphate buffer	59	4.37	4.23
EMS (0.025 M)	63	11.01	9.73
As-contaminated groundwater	63	16.19	9.10

[a] See footnotes to Table 4 for details.

[b] Not arcsine-transformed.

mixtures much as it did to EMS, a known mutagen. This response was observed as increases in the percentage of aberrant anaphases and in the number of aberrations per hundred anaphases.

Our results with the barley root tip cytogenetic aberration assay agree with expectations. According to Brewen and Preston (1978), structural changes in chromosomes constitute a significant proportion of mutagenic events. Chemicals that induce mutations in eukaryotes invariably also induce chromosomal structural changes (Evans, 1976; Kunzel, 1971). Fly ash from coal combustion is mutagenic in bacteria; known mutagens have been isolated and identified. Arsenic and heavy metals are known to induce both mutations and cytogenetic effects.

Chrisp et al. (1978) reported that horse serum, phosphate-buffered saline, and cyclohexane filtrates of fly ash of 2.2-μm mass median diameter (the finest particle size fraction tested) induced histidine revertants in S. typhimurium strains TA98 and TA1538. The order of activity was horse serum >> cyclohexane > saline. More recently, Fisher et al. (1979) reported that serum filtrates of the most respirable stack-collected fly ash are mutagenic in S. typhimurium strains TA98, TA100, and TA1538. However, after heating to 350°, these serum filtrates are not mutagenic in the Salmonella assays. The authors hypothesized that the mutagenic activity of fly ash is associated with organic compounds. Lee et al. (1980) have found dimethylsulfate and its hydrolysis product monoethylsulfate at concentrations as high as 830 ppm in fly ash and airborne particulate matter from coal combustion. These compounds are known mutagens.

In the case of the As-contaminated groundwater, numerous metals were present (e.g., in μg/l: cadmium, 485; nickel, 935, lead, 117; antimony, 297; thallium, 7720, and zinc, 251; Epler et al., 1980). Arsenic, especially in the arsenite state, is known to induce mutations and chromosome aberrations (Rossner, 1977). Some of the heavy metals are known to be mutagenic and/or carcinogenic (Freese, 1971; Miller and Miller, 1971).

CONCLUSIONS

The following conclusions were reached: 1) The *Salmonella* and *Saccharomyces* assays indicated the presence of mutagenic activity in the XAD-2 concentrate of the As-contaminated groundwater but not in the aqueous extract of the fly ash sample. 2) Both assays implicated frameshift mutagenesis as the mechanism involved. 3) The *Hordeum* root tip assay indicated mutagenic activity in both complex mixtures tested. 4) Chemical analyses of both complex mixtures showed the presence of heavy metals, implicating them as the possible cause of chromosomal aberrations.

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SESSION 4

MOBILE SOURCES

PB82-233362

SHORT-TERM CARCINOGENESIS AND MUTAGENESIS BIOASSAYS OF MOBILE-SOURCE EMISSIONS

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INTRODUCTION

The combustion emissions from mobile sources, including both gases and particles, are very complex and may have thousands of separate components. Qualitative and quantitative identification of all of these individual components is a tremendous task. The analytical challenge is facilitated if the number of compounds requiring identification can be reduced.

Short-term bioassays can be used to narrow the compounds requiring identification to those potentially responsible for adverse health effects. Initial screening of complex mixtures is useful to

1) indicate particular emissions or portions of an emission that are potentially toxic, mutagenic, or carcinogenic and that should be evaluated in confirmatory and, possibly, long-term bioassays;

2) biologically direct the fractionation and identification of hazardous components and specific chemicals in complex mixtures; and

3) compare the relative biological activity of similar emissions that result from different sources, fuels, control technologies, or operating conditions.

The introduction of increasing numbers of light-duty diesel automobiles has stimulated environmental concern over the health effects of diesel particulate emissions. Currently, diesel

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automobiles emit over one hundred times the particles (grams per mile) emitted by gasoline-powered, catalyst-equipped (gasoline-catalyst) automobiles. Diesel particles, emitted as carbonaceous soot, serve as condensation nuclei for higher-molecular-weight organic combustion vapors, which condense onto the soot particles as the exhaust is diluted and cooled to ambient temperature. The diesel particles emitted into the ambient air contain 10 to 50% extractable organic constituents.

The gaseous organics that do not adsorb onto particles are currently regulated only as total hydrocarbon emissions. Components of this general class of emissions that are of potential concern, such as aldehydes, nitrosamines, phenols, and cyanides, are not specifically regulated. Research to apply short-term bioassays to these gaseous emissions is being initiated; much of the research completed to date, however, has focussed on the organic compounds extracted from diluted particulate emissions.

APPLICATION OF MICROBIAL ASSAYS TO MOBILE SOURCE EMISSIONS

Mutagenic activity resulting from organics extracted from diesel particulate emissions was first detected using microbial mutagenesis assays. Particles collected from two heavy-duty diesel engines were subjected to extraction and fractionation techniques (Huisingh et al., 1979). The resulting organic fractions (acidic, basic, and neutral) were then screened using bioassays that employed bacteria (*Salmonella typhimurium*) to detect gene mutations and mammalian cells to detect cellular toxicity. None of the diesel organic fractions was found to be highly cytotoxic in the mammalian cell assays. All but one of the fractions showed some mutagenicity in the *S. typhimurium* plate-incorporation assay for gene mutations.

The neutral components of the diesel extract accounted for 84% of the mass and were fractionated into four subfractions (paraffins, aromatics, and transitional and oxygenated polar neutrals). The paraffinic fraction (39% by weight) was not mutagenic, and the aromatic fraction (13% by weight) accounted for only 1.5% of the mutagenic activity in the TA98 strain of *S. typhimurium*. The two polar neutral fractions, transitional and oxygenated, were the most mutagenic. These two fractions accounted for one third of the mass of the extractable organics and over 90% of the mutagenic activity in both TA98 and TA1538 strains of *S. typhimurium.*

These results suggest that there is more than one mutagen present in the polar neutral fractions of organics bound to diesel particles. These mutagens are not artifacts of the extraction or fractionation processes (Huisingh et al., 1979), but appear to be products of the combustion process, since fractions of uncombusted fuel were not mutagenic.

Various fuels appear to differ in the mutagenicity of their particle-bound combustion organics. Studies comparing the mutagenic activity of combustion emission organics from two passenger cars operated with five different fuels show that the poorest quality fuel (No. 2 diesel fuel) generated the largest quantity of mutagenic particle-bound organics (Huisingh et al., 1979). This minimum-quality fuel had the lowest Cetane index (41.8), highest aromatic content, and highest nitrogen and sulfur contents.

The effects of engine, fuel, and operating conditions on the mutagenicity of automotive emissions were studied using short-term bioassays. These conditions are variable and may affect not only the mutagenic activity of the organic fractions but also the amount of extractable organics present on the diesel particles and the particulate emission rate. These factors can be accommodated by calculations that determine the mutagenic activity on a per-mile- or per-kilogram-fuel-consumed basis.

In comparing different diesel automobiles, Claxton and Kohan (1980) found as much as a three-fold difference in their mutagenic emission rate. Although the extractable organics from the gasoline-catalyst automobile emissions were more mutagenic than many of the diesel organics, the amount of extractable organics and the particle emission rate were so low for the gasoline-catalyst automobile that the net mutagenic activity per mile was two orders of magnitude less than that from a comparable diesel automobile.

The total mutagenic activity resulting from automotive emissions depends on the release of the mutagenic organics from the particles. The ability of physiological fluids (serum, lung-cell cytosol, and lung-lavage fluid) to release mutagens from diesel particles has been compared with the extraction capability of solvents. Serum and lung cytosol were found to remove 80 to 85% of the solvent-extractable mutagenic activity from the diesel particles (King et al., in press). The serum- and cytosol-associated mutagens were essentially undetectable when the serum itself was tested in the *S. typhimurium* mutagenesis bioassay. This effect is possibly due to binding of the mutagens by the serum. Other studies have shown that whole diesel particles are engulfed by mammalian cells *in vitro* and are capable of causing gene mutations (Chescheir et al., 1980).

APPLICATION OF MAMMALIAN CELL BIOASSAYS TO MOBILE-SOURCE EMISSIONS

The extractable organics from diesel particles, although showing a low cellular toxicity in the microbial bioassays, were mutagenic in a microbial (*S. typhimurium*) assay and positive in a yeast (*Saccharomyces cerevisciae*) assay for DNA damage (mitotic recombination). These results indicated the presence of potentially mutagenic or carcinogenic chemicals in diesel emission organics (Huisingh et al., in press b).

Mammalian cell bioassays were initiated to verify the microbial screening results; mammalian cells are much more similar to human cells in cellular and chromosomal organization than are microbes. Diesel organics gave positive results in two forward mutational assays using mammalian cells. Two assays for DNA damage--unscheduled DNA synthesis (UDS) and sister chromatid exchange (SCE) assays--were also used. The UDS assay was negative and the SCE assay positive with the diesel organics tested. The carcinogenesis assay for morphological oncogenic transformation in the mammalian (BALB/c 3T3) cells was positive.

Additional research is needed to determine which bioassays are most useful in evaluating automotive emissions and to develop new methods to expose these test systems to "difficult" samples, such as gases and insoluble organics.

COMPARATIVE BIOASSAYS OF MOBILE SOURCE EMISSIONS

A matrix of *in vitro* and *in vivo* bioassays is currently being used to quantitatively compare the effects of a series of mobile-source emissions (extractable organics from particulate emissions: Huisingh et al., in press a). The normalized rankings for four bioassays are compared in Table 1. The quantitative results from the mobile-source samples show a general overall consistency (Nesnow and Huisingh, in press). The Cat sample was very weak in all of the assays. The Nissan sample showed the highest activity in these assays, while the other three mobile sources showed intermediate activity.

In theory, gene mutation and skin tumor initiation arise from similar mechanisms and, thus, should give similar results (assuming equal toxicity and mutagen or carcinogen transport and activation by the various cell types). A comparison of the results of the microbial and mammalian cell mutation assays with the results of the rodent skin tumor initiation assay seems to support this hypothesis.

Table 1. Activity Rankings for Mobile-source Emissions[a,b]

Activity	Heavy-duty Diesel Cat	Light-duty Diesel Nissan	Light-duty Diesel Olds	Light-duty Diesel VW Rab	Gasoline-catalyst Mustang
Microbial mutation[c]	4.3	100	23	22	25
Sister chromatid exchange[d]	0	100	0	50	1
Mammalian cell mutation[e]	1	100	64	50	36
Rodent skin tumor initiation[f]	0	100	45	1	35

[a] All data are expressed as a percentage of the Nissan diesel activity, which was assigned a value of 100.

[b] Cat is the Caterpillar 3208, 4-stroke cycle engine: Olds is Oldsmobile; VW Rab is Volkswagen Rabbit.

[c] *S. typhimurium* histidine reversion assay; TA98 with S-9 activation (Aroclor-induced).

[d] Chinese hamster ovary cell assay with Aroclor-induced S-9 activation.

[e] L5178 mouse lymphoma forward mutation asssay at the thymidine kinase locus with Aroclor-induced S-9 activation.

[f] SENCAR mouse assay using TPA (12-0-tetradecanoylphorbol-13-acetate as the tumor promoter.

Other comparative source samples (roofing tar, coke oven emissions, and cigarette smoke condensate) were also evaluated in this study (Huisingh et al., in press a; Nesnow and Huisingh, in press). The quantitative results for these samples, which required metabolic activation, showed less agreement between these bioassays. Thus, it may not be possible to quantitatively extrapolate from *in vitro* to *in vivo* results for all types of complex mixtures.

CONCLUSIONS

Short-term carcinogenesis and mutagenesis bioassays, now being widely applied to the evaluation and characterization of mobile source emissions, show that the organics associated with both diesel and gasoline-catalyst particulate emissions exhibit mutagenic and carcinogenic activity. The relative potency of different mobile sources varies significantly.

Current research is focussing on the following areas: 1) comparative potency of the emissions from a variety of mobile sources, 2) comparative evaluation of a battery of bioassays for mobile-source applications, 3) identification of the hazardous components in diesel emissions, and 4) determination of the effective dose and target for those hazardous components.

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TUMORIGENESIS OF EMISSION EXTRACTS ON MOUSE SKIN

TUMORIGENESIS OF DIESEL EXHAUST, GASOLINE EXHAUST, AND RELATED EMISSION EXTRACTS ON SENCAR MOUSE SKIN

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INTRODUCTION

Recent advances in the study of particulate emissions have brought to light several facts concerning their health effects. Many emission sources produce respirable particles with associated organic substances (Waters et al., 1979). These organic substances may be unburned fuel or they may result from pyrosynthetic reactions at or near the combustion source and photosynthetic and oxidative processes that occur after their initial formation (Crittenden and Long, 1976). Some of these organic materials contain known carcinogens and are mutagenic in short-term bioassays (Huisingh et al., 1979). Previous work by Kotin et al. (1966) and by Mittler and Nicholson (1957) gave conflicting results on the mouse skin tumorigenicity of diesel exhaust components. Similar studies with gasoline exhaust revealed a positive tumorigenic response from multiple application of condensates and extracts to mouse skin (Kotin et al., 1964; Mittler and Nicholson, 1957; Hoffmann and Wynder, 1963; Hoffmann et al., 1965). The present study was performed to examine the tumorigenicity of the organics associated with diesel exhaust particulate emissions using a sensitive mouse skin tumorigenesis model (SENCAR) and to compare the tumorigenic potency of the organics from particulate emissions of diesel, gasoline, and related emission sources.

The SENCAR mouse is a relatively new stock of carcinogen-sensitive animals, which up to this time has not been used extensively in bioassay programs. A description of the SENCAR system and of mouse skin tumorigenesis in general follows, to explain the strengths and weaknesses of this short-term *in vivo* carcinogenesis bioassay.

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The SENCAR mouse stock has been selected for its increased sensitivity to two-stage carcinogenesis using 7,12-dimethylbenz(a)-anthracene (DMBA) as the initiator and 12-O-tetradecanoylphorbol-13-acetate (TPA) as the promotor. This system is also more sensitive to other polycyclic aromatic hydrocarbons (PAH) such as benzo(a)pyrene (B[a]P) (Slaga et al., in press a). In addition to its well-documented response to PAH (Slaga et al., 1978b), the mouse skin tumorigenesis bioassay system has identified many chemicals other than PAH as potential carcinogens (Table 1). These chemicals represent a wide variety of structural classes, including aldehyde, carbamate, epoxide, haloalkyether, haloaromatic, haloalkylcarbonyl, hydroxylamine, lactone, nitrosamide, sulfonate, sultone, and urea. This list of 32 chemicals includes such well-known chemical carcinogens as aflatoxin B1, bis(chloromethyl)ether, chloromethyl methyl ether, urethane, N-acetoxy-2-acetamidofluorene, β-propiolactone, N-methyl-N'-nitro-N-nitrosoguanidine, 1,3-propanesultone, N-nitrosomethyl urea, triethylenemelamine, and 4-nitroquinoline-N-oxide. The mouse skin tumorigenesis bioassay can also detect chemicals that cause tumors in the respiratory tract of animals (Table 2). Of 11 known animal respiratory carcinogens, the mouse skin tumorigenesis system has to date detected PAH, quinolines, and carbamates. Of 11 highly suspect occupational respiratory carcinogens, the mouse skin tumorigenesis system has to date detected chloromethyl ethers and coke oven emissions. These results indicate that the mouse skin tumorigenesis bioassay can detect both dermal and nondermal carcinogens.

The two basic protocols that can be employed to detect chemical carcinogens in the mouse skin tumorigenesis assay are illustrated in Figure 1. Multiple application of the test agent for up to 60 weeks will give rise primarily to malignant carcinomas of the skin. This protocol for complete carcinogens is a test for agents exhibiting both tumor-initiating and tumor-promoting activities. The bioassay protocol for tumor initiators is a single application of test agent followed one week later by multiple applications of a potent tumor promoter. Tumor initiation is one step in the multistep carcinogenic process and involves the conversion of a normal cell into a preneoplastic one. In the case of chemical carcinogens, it involves the interaction of chemicals or their activated forms with cellular DNA. These initiated cells remain dormant for periods of up to one year, or until they are stimulated to progress into hyperplastic or neoplastic lesions. This stimulation is called tumor promotion and is accomplished by applying croton oil or its most active component, TPA. An initiated cell is, therefore, an irreversibly formed preneoplastic lesion that can be stimulated to express the transformed phenotype.

Table 1. Chemicals Other Than PAH Detected by Mouse Skin Bioassay

Class	Chemical	Reference
Aldehyde	Malonaldehyde	Shamberger et al., 1974
Carbamate	Urethane	Salaman and Roe, 1953 Slaga et al., 1973
	Vinyl carbamate	Dahl et al., 1978
	Ethyl N-phenylcarbamate	Roe and Salaman, 1955
Epoxide, diepoxide	Glycidaldehyde	Shamberger et al., 1974 Van Duuren et al., 1965
	1,2,3,4-Diepoxybutane	Van Duuren et al., 1965
	1,2,4,5-Diepoxypentane	Van Duuren et al., 1965
	1,2,6,7-Diepoxyheptane	Van Duuren et al., 1965
	Chloroethylene oxide	Zajdela et al., 1980
Haloalkylether	Bis(chloromethyl)ether	Van Duuren et al., 1969 Zajdela et al., 1980 Slaga et al., 1973
	Chloromethyl methyl ether	Slaga et al., 1973 Van Duuren et al., 1969
Haloaromatic	2,3,4,5-Tetrachloronitrobenzene	Searle, 1966
	2,3,4,6-Tetrachloronitrobenzene	Searle, 1966
	2,3,5,6-Tetrachloronitrobenzene	Searle, 1966
	Pentachloronitrobenzene	Searle, 1966
Haloalkylcarbonyl	Chloroacetone	Searle, 1966
	3-Bromopropionic acid	Searle, 1966
Hydroxylamine	N-Acetoxy-4-acetamidobiphenyl	Scribner and Slaga, 1975
	N-Acetoxy-2-acetamidofluorene	Scribner and Slaga, 1975 Slaga et al., 1978b
	N-Hydroxy-2-aminonaphthalene	Clayson and Garner, 1976
	N-Acetoxy-2-acetoamidophenanthrene	Scribner and Slaga, 1975
	N-(4-Methoxy)benzoyloxypiperidine	Scribner and Slaga, 1975
	N-(4-Nitro)benzoyloxypiperidine	Scribner and Slaga, 1975
	N-Acetoxy-4-acetamidostilbene	Scribner and Slaga, 1975
Lactone	β-Propiolactone	Roe and Salaman, 1955 Slaga et al., 1973 Hennings and Boutwell, 1969
Multifunctional	Triethylenemelamine	Roe and Salaman, 1955
	4-Nitroquinoline-N-oxide	Hennings and Boutwell, 1969
Natural products	Aflatoxin B1	Lindenfelser et al., 1974
Nitrosamide	N-Methyl-N'-nitro-N-nitrosoguanidine	Hennings et al., 1978 Fujii, 1976
Sulfonate	Allyl methylsulfonate	Roe, 1957
Sultone	1,3-Propanesultone	Slaga et al., 1973
Urea	N-Nitrosomethylurea	Graffi and Hoffman, 1966

Table 2. Response of Carcinogens in Humans, Animals, and Mouse Skin

Sample	Occupational Respiratory Carcinogen[a]	Animal Respiratory Carcinogen[a]	Mouse Skin Tumorigen[b]
Arsenic	+		
Asbestos	+	+	
Beryllium	+	+	
Carbamates		+	+
Chloromethyl ethers	+	+	+
Chromium	+		
Coke oven	+		+
Isopropyl oil	+		
MOCA[c]	+	+	
Mustard gas	+	+	
Nickel	+	+	
Nitrosamines		+	
PAH		+	+
Quinolines		+	+
Vinyl chloride	+	+	

[a] Frank, 1978.

[b] Slaga et al., 1978b, in press: Van Duuren, 1976.

[c] Methylene bis(ortho-chloroaniline).

Relationships between tumor initiators and complete carcinogens have been previously described. Various structurally diverse chemicals (Table 3) are both complete carcinogens and tumor initiators in mouse skin from CD-1 and the genetically related SENCAR mouse. Some agents, however, appear to have only tumor-initiating activities in mouse skin (Table 4). The correlation between potencies as complete carcinogens and as tumor initiators is excellent for the 12 chemicals that show both kinds of activity (Table 5). The relationship between the production of papillomas and the production of carcinomas in the same animals treated with the skin tumor initiator DMBA or B(a)P is shown in Table 6. These results indicate that the number of papillomas per mouse at 15 to 20 weeks correlates well with the number of malignant carcinomas formed at 50 weeks, for animals treated with these two strong skin tumor initiators.

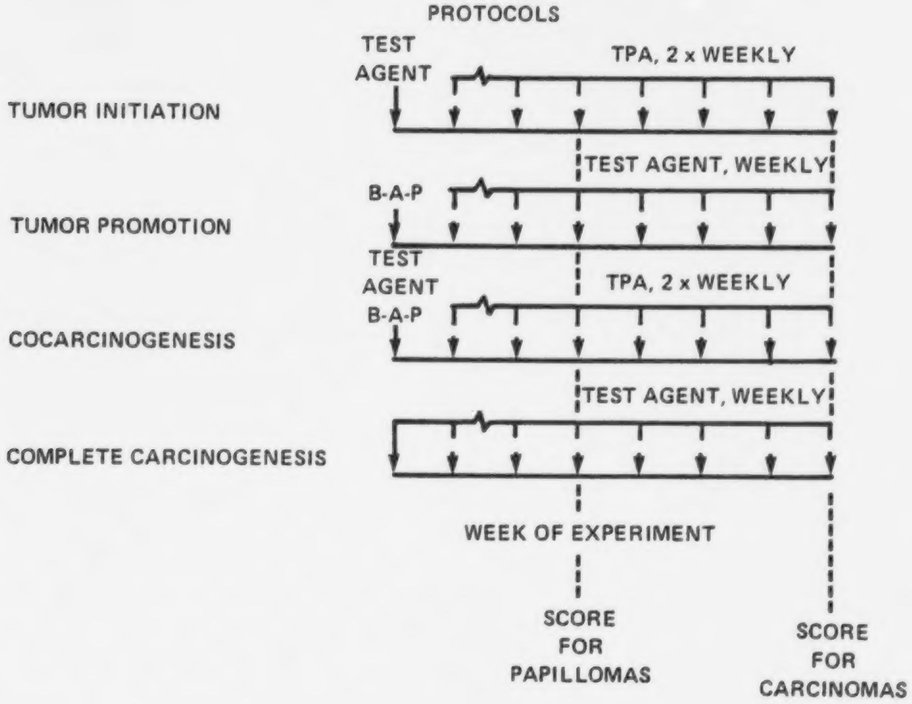
PROTOCOLS
TEST AGENT
TPA, 2 x WEEKLY
TUMOR INITIATION
TEST AGENT, WEEKLY
B-A-P
TUMOR PROMOTION
TEST AGENT
B-A-P
TPA, 2 x WEEKLY
COCARCINOGENESIS
TEST AGENT, WEEKLY
COMPLETE CARCINOGENESIS
WEEK OF EXPERIMENT
SCORE FOR PAPILLOMAS
SCORE FOR CARCINOMAS

Figure 1. Protocols for bioassays of test agents as tumor initiators, tumor promoters, cocarcinogens, and complete carcinogens.

Table 3. Compounds That Are Both Complete Carcinogens and Tumor Initiators in CD-1 and SENCAR Mouse Skin[a]

7,12-Dimethylbenz(a)anthracene
3-Methylcholanthrene
Benzo(a)pyrene
7-Methylbenz(a)anthracene
Dibenz(a,h)anthracene
5-Methylchrysene
β-Propiolactone
Bis(chloromethyl)ether
2-Hydroxybenzo(a)pyrene
Benzo(a)pyrene-7,8-oxide
Benzo(a)pyrene-7,8-diol
7,12-Dimethylbenz(a)anthracene-3,4-diol

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[a] Hecht et al., 1979; Slaga et al., 1978b, in press b.

Table 4. Agents That May Be Pure Tumor Initiators in Mouse Skin[a]

Benzo(a)pyrene-7,8-diol-9,10-epoxide	Dibenz(a,c)anthracene
N-Methyl-N'-nitro-N-nitrosoguanidine	Chrysene
Benz(a)anthracene-3,4-diol-1,2-epoxide	Urethane
Benz(a)anthracene	Triethylenemelamine

[a] Scribner, 1973; Scribner and Slaga, 1975; Slaga et al., 1973, 1978a, 1979; Van Duuren, 1976.

Table 5. Comparison of Complete Carcinogenesis and Tumor Initiation in Mouse Skin

Compound	Relative Potency[a]	
	Complete Carcinogenesis (carcinomas)	Tumor Initiation (papillomas)
7,12-Dimethylbenz(a)anthracene	100	100
3-Methylcholanthrene	50	50
Benzo(a)pyrene	30	30
2-Hydroxybenzo(a)pyrene	30	30
7-Bromomethyl-12-methylbenz(a)anthracene	20	20
Benzo(a)pyrene-7,8-oxide	20	20
Dibenz(a,h)anthracene	20	20
Benz(a)anthracene	5 ± 5	5
Dibenz(a,c)anthracene	0	3
Pyrene	0	0
Benzo(a)pyrene-4,5-oxide	0	0
Anthracene	0	0

[a] Relative potency was determined from dose-response data. DMBA was given a maximum value of 100 (Slaga et al., in press b).

Table 6. Dose-response Studies on the Ability of DMBA and B(a)P to Initiate Skin Tumors in SENCAR Mice[a]

Initiator	Dose (nmol)	No. of Papillomas per Mouse at 15 Weeks[b]	% of Mice With Papillomas at 15 Weeks	% of Mice With Carcinomas at 50 Weeks[b]
DMBA	100.0	22.0 (100)	100	100
DMBA	10.0	6.8 (32)	100	40
DMBA	1.0	3.2 (15)	93	22
DMBA	0.1	0.5 (2)	20	5
B(a)P	200.0	7.5 (100)	100	55 (100)
B(a)P	100.0	3.2 (43)	78	30 (55)
B(a)P	50.0	1.4 (19)	60	18 (33)

[a] Mice were treated one week after initiation with twice weekly applications of 5 μg TPA.

[b] Values in parentheses represent percent normalized to the highest dose tested of each agent (Slaga et al., in press b).

MATERIALS AND METHODS

Sample Generation and Isolation

The details of sample generation and isolation have been reported elsewhere (Huisingh et al., in press). Briefly, the mobile-source samples consisted of particulate emissions from two diesel-fueled vehicles, one gasoline-fueled vehicle, and one diesel engine (Table 7): a heavy-duty Caterpillar 3304 engine mounted on an engine dynamometer at 2200 rpm steady state with an 85-lb load; a Datsun-Nissan 220-C; an Oldsmobile 350; and a 1978 Mustang II-302 V-8 catalyst engine (with emission controls and using unleaded gasoline) mounted on a chassis dynamometer with a repeated highway fuel economy cycle of 10.24 mi, an average speed of 48 mph, and a running time of 12.75 min. The Caterpillar, Datsun-Nissan, and Oldsmobile engines were fueled with the same batch of No. 2 diesel fuel. Particulate samples were collected using a dilution tunnel in which the hot exhaust was diluted, cooled, and filtered through Pallflex Teflon-coated fiberglas filters.

The comparative sources employed were cigarette smoke condensate, coke oven samples, and roofing tar emissions. Cigarette smoke condensate was obtained by condensing smoke from an 85-mm nonfilter Kentucky reference cigarette 2R1. Condensate was collected in acetone and refrigerated in Dry-Ice-isopropanol bath. Cigarette smoke condensate acetone suspension was adjusted with

Table 7. Mobile Source Sample Generation

Sample	Description	Fuel	Driving Cycle
Diesel			
Cat	Caterpillar 3304	Diesel No. 2	Mode II[a]
Nissan	Nissan Datsun 220C	Diesel No. 2	HWFET[b]
Olds	Oldsmobile 350	Diesel No. 2	HWFET
Gasoline			
Mustang	1978 Mustang, II-302, V-8 catalyst and EGR	Unleaded gasoline	HWFET

[a] Mode II cycle was conducted at 2200 rpm steady state with an 85-lb load.

[b] Highway fuel economy cycle (HWFET) was a 10.24-mi cycle averaging 48 mph and taking 12.75 min.

appropriate amounts of acetone and water. Coke oven samples were collected from the top of a coke oven battery at Republic Steel, Gadston, AL, using the Massive Air Volume Sampler. Due to local wind conditions, various types of aerosols were sampled; thus, an unknown but significant portion of the emission sample may have been from the urban environment. The roofing tar emission sample was collected using a conventional tar pot with external propane burner. Pitch-based tar was heated to 360 to 380°F (182 to 193°C), and emissions were collected using a 6-ft (1.8-m) stack extension and Teflon socks in a baghouse.

The mobile source, coke oven, and roofing tar emission samples were Soxhlet-extracted with dichloromethane. The dichloromethane was removed by evaporation under dry nitrogen, and the samples were shipped in coded form in dry ice to Oak Ridge National Laboratories where the animal experiments were conducted. Table 8 shows the amount of organic material extracted from the particles with dichloromethane and the amount of B(a)P per milligram extract or per milligram particle in each sample. The B(a)P analysis was performed according to the method of Snook et al. (1976) or Swanson et al. (1978). Percent extractable of organic material from the particles varied from 8% of the Nissan sample to a maximum of 99% for the roofing tar sample. Since cigarette smoke condensate was not a particulate sample per se, the complete sample was used in

Table 8. Benzo(a)pyrene Analysis[a]

Sample	Extractable (%)	B(a)P (ng/mg extract)	B(a)P (ng/mg particle)
Diesel:			
Cat	27	2	0.5
Nissan	8	1173	96.2
Olds	17	2	0.4
Gasoline:			
Mustang	43	103	44.1
Comparative Sources:			
Cigarette	--	<1	--
Coke	7	478	31.5
Roofing tar	>99	889	889

[a] B(a)P analysis was performed according to Swanson et al. (1978), except for analysis of cigarette smoke condensate, which was performed according to Snook et al. (1976).

the biological analysis. B(a)P in the extracts varied from less than 1 ng/mg extract for the cigarette sample to a high of 1173 ng/mg extract for the Nissan sample.

Animals

SENCAR mouse stock, selected for its increased sensitivity to carcinogenesis (Boutwell, 1964) was used in this study. These mice were derived by breeding Charles River CD-1 mice with male STS (skin-tumor-sensitive) mice that were originally derived from Rockland mice. Mice were selected for sensitivity to the DMBA-TPA two-stage system of tumorigenesis for eight generations. These mice were initially obtained from Dr. R. Boutwell (McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, WI) and now are being raised at the Oak Ridge National Laboratory, Oak Ridge, TN.

Chemicals

TPA was obtained from Dr. P. Borchert (University of Minnesota Minneapolis, MN) and B(a)P from Aldrich Chemical Co. All the agents were prepared under yellow light immediately before use and applied topically in 0.2 ml of spectral-quality acetone.

Tumor Experiments

These studies employed 80 mice per treatment group (40 of each sex). All the mice were shaved with surgical clippers two days before the initial treatment, and only those mice in the resting phase of the hair cycle were used. Five dose levels were used for the tumor-initiating activities of the various samples, except for the Mustang sample, which was tested at four dose levels. B(a)P was used as the standard for the tumor-initiation studies, using four dose levels. One week after application, the tumor promoter TPA was administered twice weekly. All samples at all doses were applied as a single treatment, except for the 10-mg dose, which was administered in five daily doses of 2 mg. Skin tumor formation was recorded weekly, and papillomas greater than 2 mm in diameter were included in the cumulative total if they persisted for one week or longer. Both the number of mice with tumors and the number of tumors per mouse were determined and recorded weekly. Papillomas and carcinomas were removed randomly for histological verification.

RESULTS AND DISCUSSION

The organic extracts from particulate emissions described previously were applied to the backs of SENCAR mice according to the protocols cited in Materials and Methods. The production of benign papillomas on a weekly basis is depicted in Figure 2 for both the reference standard B(a)P and the Nissan sample. In both cases, after a 7-to 8-week latency period, the percent of animals bearing tumors rose dramatically between weeks 8 to 14, with a 95 to 100% tumor incidence observed in both of these dose groups. Mean number of papillomas per mouse began to rise from control between weeks 6 to 8, increasing much more slowly than did the number of animals with tumors. A plateau was reached during weeks 22 to 25. In both cases, the numbers of papillomas per animal ranged from five to six.

B(a)P exhibited a linear dose response between 2.52 and 100.92 µg (10 to 400 nmol) in both male and female SENCAR mouse skin (Figure 3). The males seemed to be more sensitive than the females to this carcinogen, although this sex difference was not evident for the complex mixture samples evaluated. The most active sample tested in this series was the coke oven extract. The response to

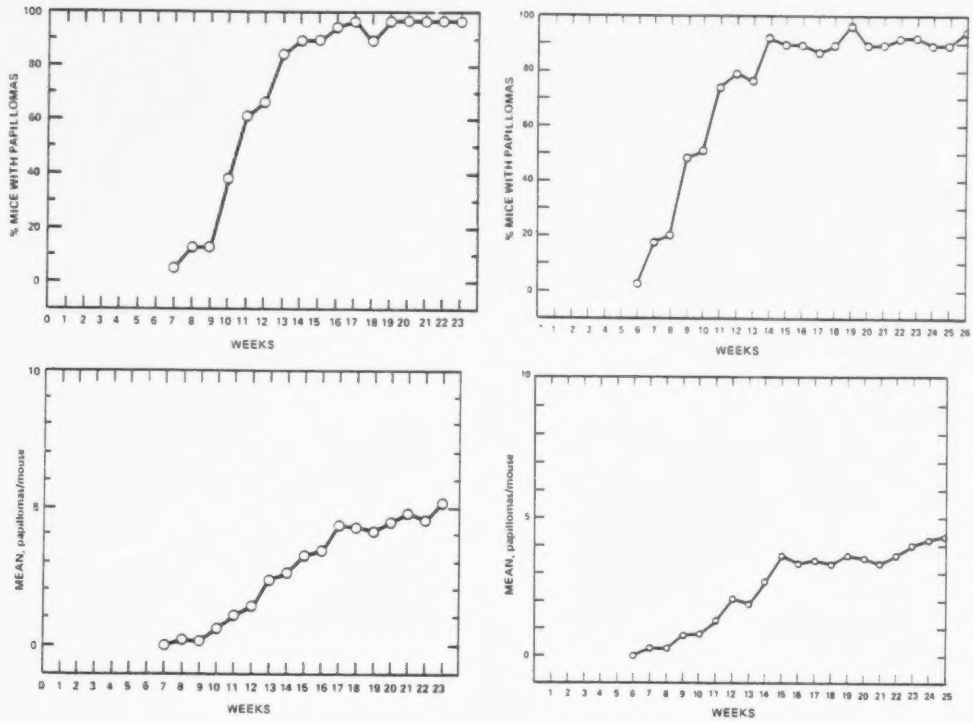

Figure 2. SENCAR mouse skin-tumor initiation. Male SENCAR (40) were initiated with either a single dose of B(a)P (50.4 μg) or five daily treatments with Nissan extract (2 mg). Animals were then treated biweekly with TPA (2 μg). Left: B(a)P. Right: Nissan extract.

this sample in both male and female animals was biphasic. An initial linear dose response was observed between 0.1 and 2 mg extract, with animals carrying an average of five to six papillomas. The roofing tar extract and Nissan extract (Figure 4) also produced a large tumor response in both male and female animals.

The Oldsmobile sample exhibited a linear dose response up to 1 mg and a subsequent loss of activity at 10 mg. The response to the Oldsmobile sample was one tenth that for the Nissan, coke oven, and roofing tar samples. The gasoline-fueled Mustang II sample also produced a weak response in both male and female animals. The "goodness of fit" (R^2) to the linear regression analysis for the female animals was extremely low, 0.686, indicating a lack of

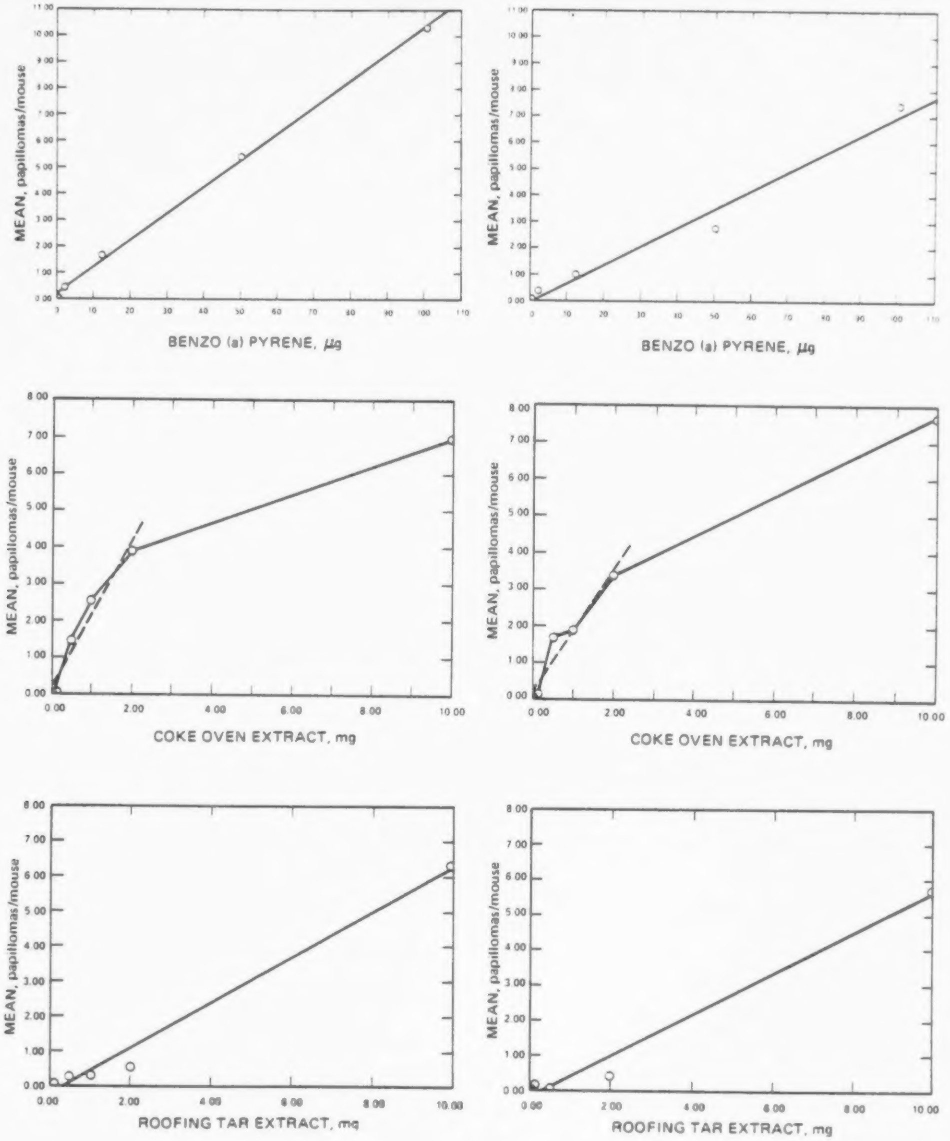

Figure 3. SENCAR mouse skin tumor initiation dose-response plots (mean number of papillomas per mouse, after subtracting background level). Graphs on left are for males and those on right are for females. There were 40 animals per dose group. The numbers of surviving animals at scoring were as follows: B(a)P--males, 156: females, 156; coke oven extract--males, 195: females 197: roofing extract--males, 197; females, 196.

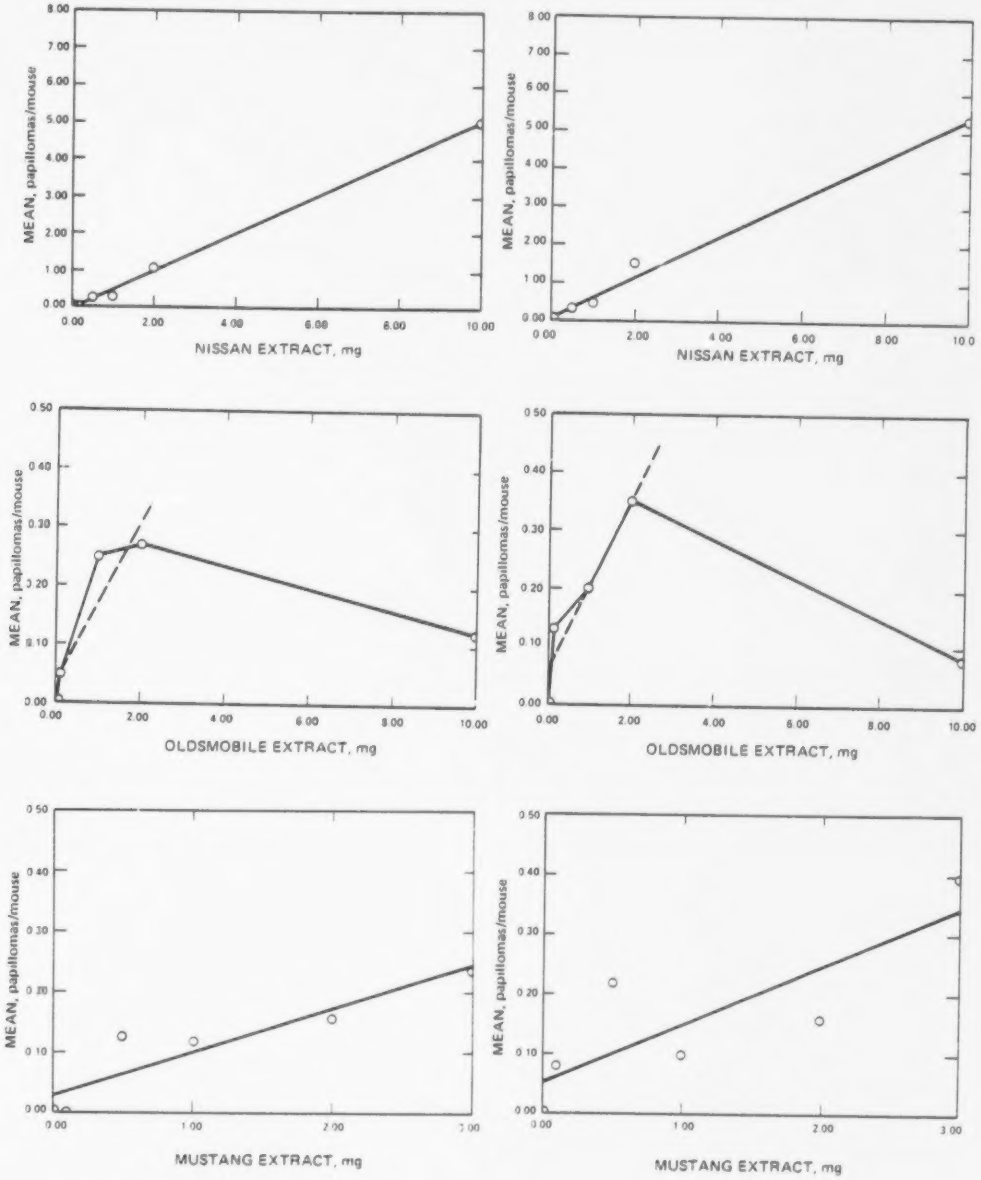

Figure 4. SENCAR mouse skin tumor initiation dose-response plots (mean number of papillomas per mouse, after subtracting background level). See Figure 3 caption for explanation. Nissan extract--males, 190: females, 198: Oldsmobile extract--males, 156: females, 157: Mustang extract--males, 188; females, 195.

linear dose response. The Caterpillar sample and cigarette smoke condensate produced two to three times the numbers of tumors found in the controls (Figure 5). However, there was no observable dose response for the doses tested (0.1 to 10 mg). The lack of activity of the cigarette smoke condensate was disappointing, although not unexpected. Cigarette smoke condensate when applied to female ICR Swiss mice twice weekly produced tumors only at relatively high doses (Gori et al., 1977; Wynder and Hoffmann, 1967). It was expected that the increased sensitivity of SENCAR mice to carcinogens would allow tumors to be observed after treatment with 10 mg whole-smoke condensate. However, this was not the case. Cigarette smoke condensate is not an extract of isolated particulates but a suspension of organics, particles, and volatiles. Therefore, it has not been concentrated to the same extent as the other samples. The detectability limit of the SENCAR mouse skin tumorigenesis assay is above the doses and concentrations tested of the cigarette smoke condensate.

The formation of spontaneous tumors in animals treated with acetone and promoted twice weekly with TPA was 0.08 and 0.05 papillomas/mouse in male and female animals, respectively, at 22 weeks after initiation; 7 to 8% of the animals had tumors. Animals initiated with up to 100.92 µg of B(a)P, followed by promotion with acetone alone, did not produce tumors.

A preliminary analysis of the results obtained was performed using a linear regression statistical analysis to produce potencies in terms of papillomas per animal per milligram agent. The results of these calculations are found in Table 9. The R^2 (goodness of fit) of the data to the linear response was greater than 0.920 for 8 out of 12 of the test groups and greater than 0.84 for 11 out of 12. Potency values ranged from 0 to 101 papillomas/mouse/mg agent. The higher of these values was obtained from the B(a)P treatment groups and was an extrapolation from the microgram dose range, where the data was obtained, to the milligram range. Obviously, this number is theoretical and based on strict linearity throughout a 1000-fold dose range, an assumption not yet proven. Also, it is a physical impossibility to have 100 papillomas on the back of a mouse. However, for comparative purposes, these values give a good approximation of the true values. A relative ranking of each of the test groups to each other after normalizing to the Nissan sample is also found in Table 9. The ranking indicates that the potency of B(a)P was greater than that of the coke oven sample, which was in turn greater than those of the roofing tar and Nissan samples. The potencies of these samples were greater than those of the Oldsmobile and Mustang samples. All of these samples were greater in potency than were cigarette smoke condensate and the Caterpillar sample, whose potencies were not significantly different from zero.

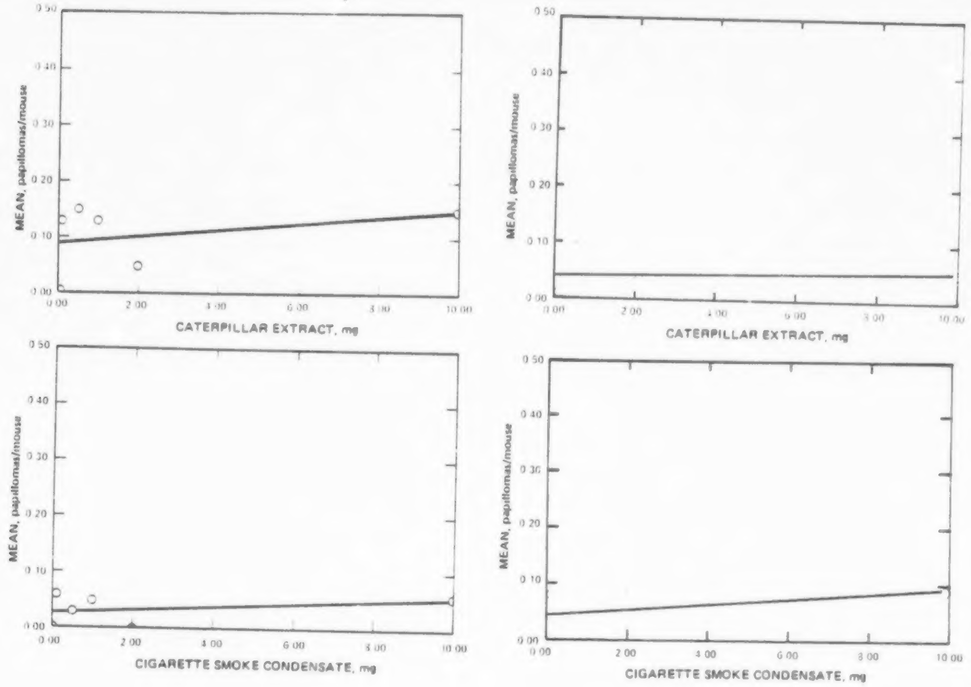

Figure 5. SENCAR mouse skin tumor initiation dose-response plots (mean number of papillomas per mouse, after subtracting background level). See Figure 3 caption for explanation. Caterpillar extract--males, 196; females 191; cigarette smoke condensate--males, 187; females, 194.

The results presented here confirm and expand the earlier observations by Kotin et al. (1966) on the tumorigenesis of diesel exhaust components and clearly indicate the tumorigenic potential of these materials. The results also indicate a range of response of diesel engines, presumably due to differences in engine technology.

Comparison of the tumor data in Table 9 with the B(a)P content per milligram extract in Table 8 indicates a lack of correlation between the two parameters. This result suggests that B(a)P and possibly other associated PAH are not reliable markers for tumorigenic activity in these complex mixtures, and that other non-PAH chemicals in the mixtures make major contributions to their overall potency.

Table 9. SENCAR Mouse Skin Tumor Initiation: Sample Rankings[a]

Sample	Papillomas/ Mouse/mg	R^2	Relative Ranking
Benzo(a)pyrene	101 (M)	0.999	20000
	71.1 (F)	0.979	13000
Coke oven	2.00 (M)	0.960	400
	1.65 (F)	0.922	310
Roofing tar	0.640 (M)	0.975	130
	0.571 (F)	0.977	110
Nissan	0.532 (F)	0.991	100
	0.507 (M)	0.998	100
Olds	0.148 (F)	0.896	28
	0.135 (M)	0.844	27
Mustang	0.097 (F)	0.686	18
	0.073 (M)	0.842	14
Cigarette	0	--	0
Caterpillar	0	--	0

[a] A linear regression model was applied to the individual data points to obtain both slope potency and R^2. M and F refer to results from male and female animals, respectively.

In conclusion, the SENCAR mouse skin tumorigenesis bioassay for tumor initiation is a quantitative short-term *in vivo* rodent carcinogenesis system that detects a variety of structurally diverse chemical carcinogens. This bioassay system has also shown its utility in evaluating complex environmental mixtures for tumorigenic potential. It gives excellent dose responses with both pure substances and complex mixtures and has shown utility for comparative potency analysis. Additional statistical models are being evaluated to analyze this data, and the results will be reported elsewhere.

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BACTERIAL MUTAGENESIS AND THE EVALUATION OF MOBILE-SOURCE EMISSIONS

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INTRODUCTION

Interest in developing a rapid, inexpensive means of detecting and evaluating the potential health hazards of mobile-source emissions is increasing. Faced with the staggering numbers of chemicals created through combustion processes that have never been assayed for mutagenicity and/or carcinogenicity, the chemist faces a futile task of identifying and controlling all potential health hazards. This study will demonstrate how bioassay techniques, and particularly the Salmonella assay, can be coupled with the fractionation of chemically complex emissions to identify components requiring more extensive analysis and control.

Emission source organics vary with factors such as time, fuel, and environmental conditions. In this study, several variables influencing mobile source studies are examined: day-to-day variation from a single source; variation among several vehicles of the same make, model, and configuration; and variation between different light-duty mobile sources. Also explored are the effects of storage and the creation of artifacts during the initial collection of sample. By understanding certain characteristics and uses of the available bacterial strains, the investigator can use microbial bioassays to aid in identification of mutagens in complex mixtures, characterize and compare the types of mutagenic components within complex mixtures, and screen various mobile sources for levels of mutagenic compounds.

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MATERIALS AND METHODS

Bioassays

The primary mutation assay used was the Salmonella typhimurium plate-incorporation assay as described by Ames et al. (1975). However, the test protocol had the following minor modifications: 1) minimal histidine was added to the base agar in the petri dish rather than to the soft-agar overlay; 2) plates were counted at 48 and 72 h to provide an additional check for toxicity factors; 3) colony counting was performed with an Artek automatic colony counter; and 4) when adequate sample was available, each dose was done in triplicate. Microsomal activation was provided using a 9000 x g supernatant of Aroclor-1254-induced Charles River CD-1 rats, as described by Ames et al. (1975).

Six indicator strains of S. typhimurium were used: TA98, TA100, TA1537, TA1538, TA1535, and TA98-FR1. The TA98-FR1 strain is a nitroreductase-deficient strain (Rosenkranz and Speck, 1975), which was provided by Dr. Herbert Rosenkranz (New York Medical College, Valhalla, NY). TA98-FR1 is deficient in only one of several nitroreductase enzymes (H.S. Rosenkranz, personal communication, 1979). All other strains were provided by Dr. Bruce Ames (University of California at Berkeley). The test results for all six indicator strains are presented in this summary paper; however, the data is given for only one strain, due to the large volume of data collected.

A second bioassay was also conducted: the 8-azaguanine forward mutation assay. Two strains of S. typhimurium, TM677 and TM35, were used, as described by Skopek et al. (1978a, b).

Samples

All samples were organic extracts from automotive exhaust particles. In each case, the vehicle was operated on a chassis dynamometer, the exhaust was diluted and cooled in a stainless steel dilution tunnel, and the particles were collected on Pallflex T60A20 glass fiber filters. The entrapped particles were then extracted with dichloromethane (Huisingh et al., 1978). The remaining organics were solvent exchanged to dimethylsulfoxide (DMSO) to a final concentration of 2 mg exhaust organics/ml DMSO. The DMSO solution was used in the bioassay. Each sample was given a unique identificaiton number to prevent bias in testing and to allow computerization of all data. These identification numbers are used for sample identification in this paper.

Sample CMBX-79-0092 is a Nissan 220C diesel vehicle, and the results for this sample are reported to demonstrate the typical response in the five major tester strains. Samples CMBX-79-0001 to CMBX-79-0012 were 12 aliquots of the same sample. Each aliquot was tested during consecutive months to ascertain any effects of storage. Multiple samples were collected on three consecutive days from the same Oldsmobile 350 vehicle and supplied as samples MSER-78-0122 to MSER-78-0135. A collection of exhaust organics from the Oldsmobile 350 diesel was chemically fractionated by the Research Triangle Institute (RTI), Research Triangle Park, NC, and was assigned numbers MSER-79-0039 to MSER-79-0046. The method of chemical fractionation is reported elsewhere (Little, 1978: Lee et al., 1976). MSER-79-0032 to MSER-79-0036 were samples from five separate gasoline automobiles of the same make, model, and configuration (Ford LTD, catalyst equipped). The variation among different makes and models of diesel automobiles was demonstrated with the TAEB samples. The nitroreductase-deficient strains were used with the following samples: an Oldsmobile 350 diesel (MSER-79-0074), a 1978 Datsun 810 gasoline (MSER-79-0064), an Oldsmobile 260 diesel (TAEB-79-0029), and a VW Dasher diesel (TAEB-79-0034) vehicle. The comparison of forward and reverse mutation systems using a preincubation protocol used an Oldsmobile 260 sample (TAEB-79-0030) and a VW Dasher sample (TAEB-79-0036). Table 1 summarizes the samples.

RESULTS

The qualitative response of the five Ames tester strains to exhaust extracts from various internal combustion engines was very consistent. TA1535 generally gave either a very low or a negative response. Each of the frameshift tester strains has given positive results. Since TA100 responds to frameshift mutagens as well as to other mutagens, it also gave a positive response. Figure 1 shows this typical response of the five tester strains to the organics from the exhaust of a Nissan 220C automobile.

Tables 2 and 3 summarize the results of bioassay data from multiple Highway Fuel Economy Test (HWFET) cycles run on three consecutive days with the same Oldsmobile 350 diesel vehicle. The bioassay data is expressed for the organics as revertants per microgram organic (slope of the linear regression line). By using the percent extractable mass and the particulate emission rate (PER) for each automobile, the revertants per gram particulate and the revertants per mile were calculated from the given slope. The revertants per microgram ranged from 2.86 to 4.33, and the revertants per mile ranged from 187,000 to 284,000. Even with the variation of test runs and bioassays, there is less than a twofold difference in the bioassay values for different filter extracts from the same vehicle operated on the HWFET cycle. The overall coefficient of variation is approximately 11%.

Table 1. Sample Information

Sample	Sample Number
Oldsmobile 350 diesel[a]	
Total particle extract	CMBX-79-0001 to 0012 MSER-78-0122 to 0135
Acid I fraction	MSER-79-0039
Acid II fraction	MSER-79-0040
Base I fraction	MSER-79-0041
Base II fraction	MSER-79-0042
Insoluble tars	MSER-79-0043
Polar neutral fraction	MSER-79-0044
Polynuclear aromatics	MSER-79-0045
Nonpolar neutral fraction	MSER-79-0046
Ford LTD gasoline automobiles[a]	
Total particle extract	MSER-79-0032 to 0036
Nissan 220C diesel[a]	
Baseline study for strains	CMBX-79-0092
Comparative vehicles[a,b]	
Oldsmobile 350 diesel[a,b]	TAEB-78-0501-0507 MSER-79-0074
Oldsmobile 260 diesel[b]	TAEB-78-0502, 0503 TAEB-79-0029, 0030
Mercedes Benz 300D diesel[b]	TAEB-78-0504, 0505
Open Record E diesel[b]	TAEB-78-0506, 0510
Chevrolet truck 350 diesel[b]	TAEB-78-0511, 0512
VW Dasher wagon diesel[b]	TAEB-78-0513, 0514
1978 Datsun 810 gasoline[a]	MSER-79-0064
VW Dasher diesel[b]	TAEB-79-0034, 0036

[a] Supplied by Ron Bradow and Roy Zweidinger, U.S. Environmental Protection Agency (EPA), Research Triangle Park (RTP), NC. Fractionation by Edo Pellizzari, RTI, RTP, NC.

[b] Supplied by Tom Baines, Emission Control Technology Division, EPA, Ann Arbor, MI.

The same diesel automobile (Oldsmobile 350) was used to generate multiple samples that were pooled subsequently for a large single sample. Twelve of the aliquots (CMBX-79-0001 to CMBX-79-0012) from this sample were stored in glass vials at -80°C in the dark. Each month for 12 months, one aliquot was tested in the microbial assay, and the results are recorded in Table 4. For

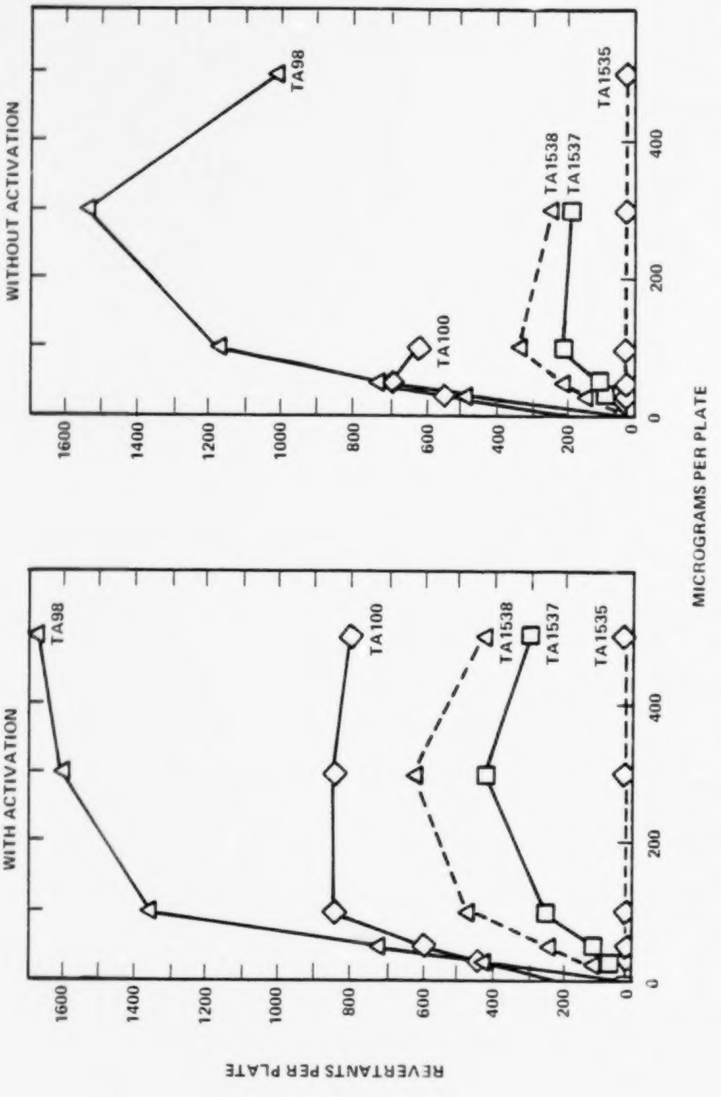

Figure 1. Exhaust organics from a Nissan 220C vehicle tested in the five tester strains of *S. typhimurium*.

Table 2. Comparative Results of Exhaust Organics from an Oldsmobile 350 Diesel Tested with *S. typhimurium* TA98 Without Activation

Sample No. (MSER-78-)[a]	Slope[b] (Rev/plate/μg)	% Ext.[c]	Rev x 10^5 /g Partic.	PER[d] (g/mile)	Rev x 10^5/mile
Day 1					
0122	4.33	10.8	4.68	0.502	2.35
0123	3.99	10.8	4.31	0.484	2.09
0124	3.82	12.4	4.74	0.495	2.34
Day 2					
0126	3.99	13.8	5.51	0.516	2.84
0129	3.86	11.4	4.40	0.517	2.28
0130	3.41	12.8	4.36	0.487	2.13
Day 3					
0132	3.72	12.6	4.69	0.518	2.43
0133	2.86	11.2	3.20	0.584	1.87
0134	3.35	11.6	3.89	0.569	2.21
0135	3.46	10.8	3.74	0.568	2.12

[a] Numbers assigned to each automobile exhaust sample collected by EPA, Environmental Sciences Research Laboratory (ESRL), RTP, NC.

[b] Slope of linear regression line.

[c] Percent dichloromethane-extractable mass.

[d] Particle emission rate (courtesy of Roy Zweidinger, EPA, ESRL, RTP, NC).

these storage samples, the revertants per microgram ranged from 4.05 to 7.23, with a mean of 5.52. The coefficient of variation for slope was 18.1%. Although obvious variance was seen from month to month, none of the differences were significant.

To test variation among vehicles of the same make, model, and configuration, five Ford LTD gasoline vehicles of the same configuration were tested. Table 5 provides the bioassay data for these vehicles. The data show that the variation among different vehicles was much greater than the variation when the same vehicle was retested. In the bioassay, the revertant per microgram organic levels varied from 1.02 to 9.61. While the various parameters of a single vehicle could vary by a factor of two, the same parameters for different individual vehicles could show a 5- to 10-fold difference. It can be seen from Table 5 that a single sample (i.e., sample MSER-79-0036) could be responsible for most of the variation, when small numbers of samples were used. When the

Table 3. Daily and Total Statistics of Results for Exhaust Organics from an Oldsmobile 350 Diesel Tested with *S. typhimurium* TA98 Without Activation

Sample No. (MSER-78-)[a]	Slope[b] (Rev/plate/μg)	% Ext.[c]	Rev x 10^5 /g Partic.	PER[d] (g/mile)	Rev x 10^5/mile
Day 1					
Mean	4.05	11.3	4.58	0.494	2.26
Standard dev.	0.26	0.9	0.23	0.009	0.15
Coeff. of var.	0.06	0.08	0.05	0.02	0.07
Day 2					
Mean	3.75	12.7	4.76	0.507	2.42
Standard dev.	0.30	1.2	0.65	0.017	0.37
Coeff. of var.	0.08	0.08	0.14	0.03	0.15
Day 3					
Mean	3.35	11.6	3.88	0.560	2.16
Standard dev.	0.36	0.8	0.62	0.029	0.23
Coeff. of var.	0.11	0.07	0.16	0.05	0.11
All days combined					
Mean	3.68	11.8	4.35	0.524	2.27
Standard dev.	0.42	1.0	0.64	0.037	0.26
Coeff. of var.	0.11	0.09	0.15	0.07	0.11

[a] Numbers assigned to each automobile exhaust sample collected by EPA, Environmental Sciences Research Laboratory (ESRL), RTP, NC.

[b] Slope of linear regression line.

[c] Percent dichloromethane-extractable mass.

[d] Particle emission rate (courtesy of Roy Zweidinger, EPA, ESRL, RTP, NC).

revertants per microgram organic were normalized to revertants per mile, the variation was reduced from a 10-fold to a 5-fold difference.

The TAEB samples (Table 6) demonstrated the degree of variation in mutagenic response found between diesel vehicles of different makes and models. Among the diesel vehicles tested, the revertants per microgram organic ranged from 1.15 to 3.23, while the revertants per mile ranged from 250,000 to 878,000.

Results from bioassay techniques can be used to guide the chemical fractionation of complex exhaust organics. This

Table 4. Mutagenicity of Diesel Exhaust Organic Samples Stored Over Varying Periods of Time Tested with *S. typhimurium* TA100

Compound	Act[a]	Dose (µg)	Mean Revertants per Plate for Monthly Samples									
			Mean	SD[b]	Mean	SD	Mean	SD	Mean	SD	Mean	SD
			JAN		FEB		MAR		MAY		JUN	
Positive control	+		734.50	62.93	741.00	43.02	778.67	36.02	669.33	10.12	993.67	43.13
	-		291.00	2.83	305.00	2.83	84.67	7.51	466.66	43.13	510.67	25.70
Negative control												
Dimethylsulfoxide	+	100.00 µl	95.00	7.07	90.00	7.07	81.67	3.21	94.00	18.08	85.67	4.73
Dimethylsulfoxide	-	100.00 µl	89.00	8.49	101.50	6.36	88.00	17.35	90.67	4.51	85.00	9.54
Oldsmobile 350 diesel	+	60.00	227.67	11.37	250.67	8.50	242.33	4.51	248.00	9.64	310.00	19.47
	+	100.00	318.33	40.50	393.33	28.02	362.50	12.02	305.00	34.39	421.33	19.22
	+	200.00	504.67	87.05	-	-	564.33	46.61	483.67	4.73	660.33	6.51
	+	600.00			-	-	1137.00	14.42	917.00	12.12	1185.00	21.07
	-	60.00	473.00	30.05	453.67	47.88	471.33	4.93	494.67	18.61	532.00	56.15
	-	100.00	633.33	29.14	591.00	78.48	628.33	27.39	605.33	12.22	739.33	20.03
	-	200.00	-	-	-	-	1077.67	20.03	934.00	8.72	1117.33	16.92
	-	600.00	-	-	-	-	1176.33	44.00	1125.00	48.12	1298.33	82.50
			JUL		AUG		SEP		OCT		NOV	
Positive control	+		674.00	49.43	118.67	21.83	338.33	20.50	1065.67	74.10	1368.00	37.72
	-		477.67	22.50	365.67	18.50	662.67	21.03	591.67	3.79	675.67	17.21
Negative control												
Dimethylsulfoxide		100.00	92.67	5.51	72.33	8.08	101.00	8.72	48.00	5.57	111.67	19.63
Dimethylsulfoxide		100.00	103.67	13.61	70.67	16.01	87.33	10.41	49.33	2.89	98.00	1.00
Oldsmobile 350 diesel	+	60.00	587.33	10.07	286.00	16.64	167.00	18.25	188.67	13.05	229.00	43.84
	+	100.00	746.67	14.29	422.33	31.56	210.33	6.03	288.33	15.95	334.33	13.65
	+	200.00	1074.67	13.58	649.33	19.40	360.33	29.40	503.67	11.02	564.00	13.23
	+	600.00	1186.33	79.05	909.00	121.62	730.00	40.34	1034.67	59.65	1186.67	70.69
	-	60.00	262.67	12.42	528.67	26.54	589.67	3.06	494.33	20.40	685.00	44.84
	-	100.00	366.00	11.14	701.00	33.96	735.33	59.80	767.33	28.57	1041.00	20.66
	-	200.00	685.67	44.38	926.33	44.74	1182.33	75.39	1230.33	16.17	1565.67	25.74
	-	600.00	1119.00	25.36	961.33	16.62	1227.33	146.17	1436.67	19.30	1920.00	59.23

[a] Metabolic activation.
[b] Standard deviation.

Table 5. Comparison of Exhaust Organics from Gasoline Vehicles of the Same Make, Model, and Configuration (Ford LTD Automobiles) in *S. typhimurium* TA98 Without Activation[a]

Sample No. (MSER-79-)		Slope[b] (Rev/plate/μg)	% Ext.[c]	Rev x 10^5 /g Partic.	PER[d] (g/mile)	Rev/ Mile
0032	Lt. blue	7.54	5.8	4.37	0.0079	3455
0033	Mid. blue	7.51	3.4	2.55	0.0100	2553
0034	Gray	9.49	3.2	3.04	0.0180	5466
0035	Silver	9.61	3.8	3.65	0.0100	3652
0036	Brown	1.02	21.4	2.18	0.0050	1091
Mean		7.03	7.52	3.16	0.0102	3243
Standard dev.		3.51	7.83	0.87	0.0048	1602
Coeff. of var.		0.50	1.04	0.28	0.47	0.49

[a] Numbers assigned to each automobile exhaust sample collected by EPA, ESRL, RTP, NC.

[b] Slope of linear regression line.

[c] Percent dichloromethane-extractable mass.

[d] Particle emission rate.

possibility was demonstrated with exhaust organic samples from an Oldsmobile 350 diesel vehicle chemically fractionated at RTI under the direction of Edo Pellizzari. When adequate sample was available, each fraction was bioassayed with all five tester strains. Results (Table 7) were similar to previous results with exhaust organics from heavy-duty diesel engines (Little, 1978). (A more complete summary is in preparation.) TA1535 gave negative results with all fractions except for the polynuclear aromatic (PNA) fraction when exogenous activation was used. The basic and nonpolar neutral fractions were negative; however, the basic fraction could not be adequately tested due to a lack of sample. The acid I, acid II, polar neutral, and PNA fractions gave positive results with each strain that responds to frameshift mutagens. This activity was demonstrated both with and without activation.

When sample CMBX-79-0047 was tested with TA98 and TA98-FR1 (Table 8), the nitroreductase-deficient strain demonstrated approximately one half the activity of TA98. With cigarette smoke condensate, both strains provided equal responses. When the results of other mobile-source organics simultaneously tested with these two strains were compared (Table 9), this relationship was not maintained.

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Table 6. Comparison of Organic Exhausts from Diesel Vehicles Using S. typhimurium TA98 Without Activation

Sample No. (TAEB-78-)	Vehicle	HC (ppm)	NO_x (ppm)	CO (ppm)	Slope[b] (Rev/plate /µg)	Mean % Ext.[c]	Rev x 10^5 /g Partic.	PER[d] (g/mile)	Rev x 10^5 /mile
501 + 0507	Olds 350	0.593	1.49	1.508	1.85	21.1	3.90	0.846	3.30
502 + 503	Olds 260	0.584	1.59	1.504	1.51	16.5	2.49	1.005	2.50
511 + 512	Chev. truck 350 diesel	0.784	1.53	1.590	2.64	53.4	14.10	0.623	8.78
504 + 505	M.B. 300D	0.251	1.38	1.377	3.23	23.3	7.53	0.840	6.32
506 + 510	Opel Record E	0.454	2.08	1.540	1.15	51.6	5.93	0.483	2.87
513 + 514	VW Dasher	0.503	0.97	1.170	1.45	53.7	7.79	0.322	2.51
Mean		0.528	1.51	1.448	1.97	36.6	6.96	0.687	4.38
Standard deviation		0.176	0.36	0.153	0.80	18.0	4.06	0.256	2.59
Coefficient of variation		0.33	0.24	0.11	0.41	0.49	0.58	0.37	0.59

[a] Provided by Tom Baines, Emission Control Technology Division, EPA, Ann Arbor, MI.

[b] Slope of linear regression line.

[c] Percent dichloromethane-extractable mass.

[d] Particle emission rate.

Table 7. Activity of Organic Fractions from an Oldsmobile 350 Diesel Exhaust Sample Tested in *S. typhimurium*[a]

Sample	Specific Activity for Strain[b]									
	TA100		TA98		TA1537		TA1538		TA1535	
	+S-9	-S-9	+S-9	-S-9	+S-9	-S-9	+S-9	-S-9	+S-9	-S-9
Acid I fraction	+	+	+	+	+	+	+	+	–	–
Acid II fraction	+	+	+	+	+	+	+	+	–	–
Base I fraction[c]	–	–	–	–	NT	NT	–	–	NT	NT
Base II fraction[c]	–	–	–	–	NT	NT	–	–	NT	NT
Insoluble tars	+	+	+	–	–	–	–	–	–	–
Polar neutrals	+	+	+	+	+	+	+	+	–	–
PNA	+	+	+	+	+	+	+	+	+	–
Non-polar neutrals	–	–	–	–	–	–	–	–	–	–

[a] Positive activity indicates a dose-dependent response with at least one dose giving a two-fold increase over spontaneous activity.

[b] NT = not tested; + = positive; – = negative.

[c] Incomplete testing.

Table 8. Mutagenicity of a Diesel Exhaust Organic and a Cigarette Smoke Condensate Tested in *S. typhimurium* TA98-FRI and TA98

Compound	Metabolic Activation	Sample (µg/Plate)	TA98-FRI (rev/plate) Mean	TA98-FRI (rev/plate) St. Dev.	Sample (µg/plate)	TA98 (rev/plate) Mean	TA98 (rev/plate) St. Dev.
Positive control							
2-Nitrofluorine	–	3.0	64.33	11.72	3.0	308.33	11.68
2-Aminoanthracene	+	0.5	1062.33	57.07	0.5	1609.00	73.26
Negative control							
Dimethylsulfoxide	–	100.0 µl	22.00	7.81	100.0	23.67	5.51
Dimethylsulfoxide	+	100.0 µl	46.00	6.24	100.0	44.00	1.73
Cigarette smoke condensate	–	60.0	27.33	2.08	30.0	25.00	9.64
	–	100.0	20.33	9.07	50.0	29.67	6.43
	–	200.0	27.00	1.73	100.0	28.33	1.15
	–	600.0	21.00	6.24	300.0	24.33	4.04
	–	1000.0	8.00[a]	–	500.0	26.00	6.56
	+	60.0	58.67	7.64	30.0	55.00	7.21
	+	100.0	67.33	12.01	50.0	68.67	6.66
	+	200.0	93.33	9.81	100.0	99.67	2.89
	+	600.0	96.33	16.17	300.0	102.67	18.18
	+	1000.0	16.00[a]	–	500.0	85.00	4.58
Nissan diesel	–	60.0	263.0	24.24	30.0	479.67	37.74
	–	100.0	421.0	25.50	50.0	729.33	31.50
	–	200.0	672.67	13.20	100.0	1175.67	10.02
	–	600.0	902.33	27.00	300.0	1543.33	73.79
	–	1000.0	575.67	76.79	500.0	1011.33	101.75
	+	60.0	231.67	27.57	30.0	421.00	55.43
	+	100.0	426.67	29.37	50.0	723.00	50.39
	+	200.0	740.00	12.17	100.0	1363.00	70.19
	+	600.0	901.00	75.36	300.0	1607.33	35.91
	+	1000.0	797.67	24.68	500.0	1680.33	38.18

Table 9. Comparison of Exhaust Organics from Different Light-Duty Automobiles with Nitroreductase-deficient (TA98-FR1) and -competent (TA98) Strains of S. *typhimurium*

Sample	Metabolic Activation	Specific Activity (rev/100 μg)	
		TA98	TA98FR1
MSER-79-0064	-	154	210
(Datsun 810 gasoline auto.)	+	341	490
MSER-79-0074	-	79	221
(Olds 350 diesel auto.)	+	81	207
TAEB-79-0029	-	46	104
(VW Dasher diesel wagon)	+	65	106
TAEB-79-0034	-	108	330
(VW Dasher diesel auto.)	+	156	257

The preliminary study comparing the response of complex mixtures in a forward and reverse mutation system was completed using four strains of S.*typhimurium*: TM677, TM35, TA100, and TA1535. Each of the four strains were used for forward mutation to 8-azaguanine resistance. Strains TA100 and TA1535 were used for reverse mutation to histidine prototrophy. Throughout this study, the preincubation assay as described by Skopek et al. (1978a, b) was used. Two samples, from an Oldsmobile 260 and a VW Dasher, were used for comparison. Table 10 gives a summary of the results. The mutagenic activity of the two samples was detected by the forward mutation system using strains TM677 and TA100: however, TA100 was not as sensitive as strain TM677. The preincubation protocol also was used for the reverse mutation assay in which strain TA100 provided a positive response. TA1535 was negative for the reversion assay.

DISCUSSION

Microbial mutagenesis assays can be used for rapid initial evaluation of combustion organics and emissions. Previously published reports (Huisingh et al., 1978) stated that most of the mutagenic activity associated with diesel samples from heavy-duty

Table 10. Comparison of Forward and Reverse Mutation Systems With the Preincubation Protocol Using Two Diesel Exhaust Samples

		8-Azaguanine Resistance (mutants/10 ^{5} survivors)				Histidine Reversion (mutants/10 ^{5} survivors)	
Sample	Dose	TM677	TA100	TM35	TA1535	TA100	TA1535
Spontaneous	-	4.4	1.2	1.2	0.6	4.1	0.2
Control: MNNG	2.04 μM	37.9	95.0	42.0	27.0	7.9	1.9
Olds 260	12.5 μg/ml	5.4	2.7	1.1	0.9	4.6	0.3
	25.0 μg/ml	6.1	4.2	1.5	1.3	6.1	0.2
	50.0 μg/ml	7.9	4.6	1.3	1.7	5.4	0.4
	100.0 μg/ml	14.6	9.3	1.2	2.0	10.8	0.3
VW Dasher	12.5 μg/ml	7.0	9.6	1.6	1.2	4.4	0.1
	25.0 μg/ml	10.7	19.1	1.6	1.8	5.9	0.1
	50.0 μg/ml	18.4	34.3	1.4	1.8	5.8	0.3
	100.0 μg/ml	34.2	43.4	1.6	1.5	9.5	0.3

engines was detected by the indicator strains that respond to frameshift mutagens. This paper provides data on light-duty diesel passenger automobiles. Each diesel and gasoline organic sample for which testing has been completed in all five strains to date produced a negligible response in TA1535, but positive responses in TA98, TA100, TA1537, and TA1538. Figure 1 shows the response of the five tester strains to Nissan 220C diesel automotive exhaust organics. Although the magnitude of response varied, the overall qualitative response was similar for all automobiles tested to date.

A great concern in testing complex environmental samples is the variability expected from multiple mixtures of chemicals. Therefore, the difficulty in sorting out the factors that cause this variability was explored. Tables 2, 3, 4, and 6 show the variability of bioassay and chemical data with different types of comparisons. Tables 2 and 3 compare the results for samples collected from one automobile at various times. Both the bioassay data and chemical data showed less than a twofold difference in all comparisons and a coefficient of variation less than 20%. A comparison of samples from vehicles of the same make, model, and configuration (Table 5) demonstrates an increased variability over multiple samples taken from one vehicle. There was nearly a tenfold difference in revertants per microgram of organic material between two automobiles. In this comparison, one automobile (the brown Ford LTD, sample MSER-79-0036) introduced significantly different values for most of the parameters measured: however, the variability for the bioassay was normalized to a large extent with the calculation of revertants per mile. Clearly, differences in emission characteristics can result in widely different mutagenic activities in emission organics. The brown Ford LTD, for example, may have emitted an unusual amount of unburned fuel or oil that would have diluted the concentration of mutagens and increased the percent extractable. However, since the amount of fuel burned for a predetermined distance would not be altered, the calculation of revertants per mile would normalize the data. Table 6 is the averaged data from replicate experiments for six different light-duty diesel vehicles. The variation among different diesel vehicles is similar to the variation between the different Ford LTD gasoline automobiles. There are, however, two dramatic differences First, the organics of the gasoline vehicles demonstrate more mutagenicity on a per weight of particulate basis. Second, when normalized to revertants per mile, there is an approximately 50- to 100-fold difference between the diesel vehicles and the Ford LTDs. The mean for the different diesel vehicles is 438,000 rev/mile, and the mean for the Ford LTDs is 3,200 rev/mile. This difference is attributable mainly to the difference in PER and demonstrates the need for adjusting the data for specific needs and comparisons. Table 11 summarizes the data from different comparisons.

Table 11. Comparison of Summary Data Demonstrating the Effect of Differing Sampling Parameters (Derived from Tables 4, 7, and 9)

	HC (ppm)	NO_x (ppm)	CO (ppm)	Slope (rev/plate/μg)	% Ext.	Rev x 10^5 /g Partic.	PER[b] (g/mile)	Rev x 10^5/mile
Different runs within same automobile (diesel)								
Mean	0.249	1.550	0.899	3.68	11.8	4.35	0.524	2.27
Standard deviation	0.032	0.10	0.070	0.42	1.0	0.64	0.037	0.26
Coeff. of var.	0.13	0.07	0.08	0.11	0.09	0.15	0.07	0.11
Vehicles of same make, model, and configuration (gasoline)								
Mean	0.23	1.74	0.37	7.03	7.52	3.16	0.0102	0.032
Standard deviation	0.20	0.66	0.10	3.51	7.83	0.87	0.0048	0.016
Coeff. of var.	0.89	0.38	0.27	0.50	1.04	0.28	0.47	0.49
Different diesel vehicles								
Mean	0.528	1.51	1.448	1.98	36.6	6.96	0.687	4.38
Standard deviation	0.176	0.36	0.153	0.80	18.0	4.06	0.256	2.59
Coeff. of var.	0.33	0.24	0.11	0.41	0.49	0.58	0.37	0.59

[a] Slope of linear regression line.

[b] Particle emission rate.

Decisions on pollution control devices, alterations in engine design, criteria for fuel characteristics, and some environmental regulations rely upon an understanding of which specific organics are likely to have a detrimental health effect. Bioassay-guided chemical fractionation has the potential to speed the identification of biologically active compounds in this category. Table 7 indicates that the chemist needs to place a higher priority on chemical identification of the polar neutral fraction than the nonpolar neutral fraction. Also, by examining the specific activity of the different fractions, one notices that the PNA fraction gives a positive response without activation. This response may be due to spillover of polar neutral chemicals into the PNA fraction with this particular fractionation scheme.

Nitroreductase-deficient strains of bacteria cannot metabolize the nitrogen components of a chemical; therefore, a decreased response in the nitroreductase-deficient strain supports the conclusion that active nitro compounds are present in the organic mixture. If activity of the compound does not depend on the reduction of the nitrogen group, activity in a nitroreductase-deficient strain should not differ from that of its parental strain. This decreased response occurred with some samples, though not all (Tables 8 and 9). This demonstrates that nitroaromatics may be one class of mutagens prominent in mobile source emissions. Whether these nitro compounds are true artifacts or are also created under normal environmental conditions is yet to be demonstrated.

The results of the 8-azaguanine forward mutation bioassay are consistent with earlier results in the Ames reversion assay. Although the Ames tester strains TA100 and TA1535 can be used for this forward mutation assay, the strains developed by Skopek et al. (1978a, b) were more sensitive and gave fewer technical problems in the performance of the assay. Although the assay detects a range of mutagens equivalent to the range detected by all five of the routinely used Salmonella strains, these forward mutation strains cannot be used in the same diagnostic manner. In other words, the forward mutation system detects a variety of mutagenic insults, whereas a specific type of DNA damage must occur to be detected by reverse mutation systems. As the forward mutation assay is more thoroughly validated, it becomes increasingly feasible to use the 8-azaguanine system for general screening and to resort to Ames tester strains for better characterization of the substance(s) being tested.

These samples demonstrate the uses of microbial mutagenesis assays for both qualitative and semi-qualitative assessment of mobile source emissions. This paper has demonstrated a means of comparing and evaluating the potential health hazard of polluting soot material from various mobile sources and from new technological developments for each of these sources. The classical chemical examination of exhaust components cannot be used effectively to quantitate and evaluate the thousands of organic chemicals derived from each combustion source. Whole-animal evaluation of multiple mobile sources and technological alterations would involve extremely slow evaluation and high costs. Although still controversial in many aspects, the Salmonella assay for mutagenicity provides a rapid, inexpensive means to assess genetically toxic effects. Recent results (Nesnow and Huisingh, in press) indicate that the plate-incorporation test correlates well with other tests for genotoxicity of combustion organics. By assuming that increased response in the plate-incorporation test represents greater potential health hazard, the Salmonella assay can be used to compare various sources, evaluate technological developments, and guide chemical characterization. Since mutation assays have some power to predict heritable effects, carcinogenesis, and teratogenesis (Hollaender and de Serres, eds., 1978), this assumption does not rely on direct correlation of Salmonella assay results with any one end point.

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COMPARISON OF THE MUTAGENIC ACTIVITY IN CARBON PARTICULATE MATTER AND IN DIESEL AND GASOLINE ENGINE EXHAUST

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INTRODUCTION

Airborne carbon particulate matter is a variable and complex mixture of components, including a variety of organic compounds. Its origin in urbanized and industrialized areas is primarily through various combustion processes. Motor vehicles are often viewed as a major source.

Talcott and Wei (1977) and Pitts et al. (1977) first showed that the Salmonella/microsome mutagenicity test, as developed by Ames et al. (1975), can be used to detect mutagenic--and thus potentially carcinogenic--activity in airborne particulate matter. Exploratory studies of motor vehicle exhaust (Tokiwa et al., 1978) and of emissions from a stationary combustion plant (Löfroth, 1978) also demonstrated the feasibility of the Salmonella assay for this purpose.

The present report summarizes some of the results obtained in an ongoing study of the mutagenicity, as detected by the Salmonella assay, of airborne particulate matter collected in the Stockholm area. The results are compared with mutagenicity data for motor vehicle exhaust samples obtained in a previous study (Löfroth, 1979) and by additional analyses of these samples.

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MATERIALS AND METHODS

Motor Vehicle Exhaust Samples

Exhaust samples from gasoline and indirect injection (IDI) diesel passenger cars were the same as those described by Löfroth (1979). They were obtained during U.S. Federal Test Procedure 1973 cycles by sampling the undiluted exhaust and by collecting the particulate matter on the filter at 25 to 50°C and also the formed aqueous condensate containing compounds that had not adsorbed onto particulate matter at the point of sampling. Particulate matter was Soxhlet-extracted overnight with acetone, and after removal of the major part of the acetone, the residue was dissolved in dimethylsulfoxide (DMSO). The aqueous condensates were treated in various ways, including extraction with n-pentane followed by removal of the pentane and dissolution of the residue in DMSO. These pentane-extracted samples, shown to contain the major part of the mutagenicity of the condensates, are the condensate samples that have been further analyzed in the present study, together with the samples of particulate matter. All samples dissolved in DMSO were stored frozen at -20°C. No detectable change in their mutagenic activity was noted over a period of about one year.

Airborne Particulate Matter

Airborne particulate matter was collected with Sierra 305 High Volume Samplers or similar equipment on 20.3- x 25.4-cm (8- x 10-in) glass fiber filters (Stora Kopparberg Special-produkter, Sweden) with a flow rate of 68 m^3/h (40 f^3/min). Sampling sites were located on the roof of a ten-story building in the northern part of the inner city of Stockholm and on the roof of a two-story house in a suburban community 22 km NNW from the center of Stockholm. Sampling was usually performed for 24 h, starting and ending at 6 to 7 AM. Night samples were collected between 10 PM and 6 AM.

To study the mutagenic activity of size-fractionated particles, samples were taken in the inner city of Stockholm. Particulate matter was collected with a Sierra 305 High Volume Sampler equipped with five stages of cascade impactors with slotted glass fiber collection substrates and the regular glass fiber filter as back-up filter. Simultaneous sampling at the same flow rate (68 m^3/h) was done using a second 305 Sampler without impactors. Sampling was done over four days; substrates and filters were changed every 24 h. Filters were extracted individually; the samples were first assayed separately and then combined and assayed as one sample. The four substrates from each impactor stage were extracted and assayed as one sample.

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Collected filters were wrapped in aluminum foil and stored at -20°C until they were extracted (between one and eight days). The filters were Soxhlet-extracted for 16 h with 250 ml acetone. The acetone was first evaporated under vacuum to about 10 ml and then under a stream of nitrogen on a heating block at $< 40°C$ to 0.3 to 0.5 ml. This residue was diluted with DMSO to a known volume, usually about 4 ml/filter. All samples were stored frozen at -20°C prior to and between mutagenicity assays.

Meteorological data were acquired from three official stations. One is located in the inner city of Stockholm, and records are made from three daily observations. Two are located at airports, in the western part of the city and north of Stockholm, and observations are made every 30 min.

Mutagenicity Assay

Mutagenicity was determined by the Salmonella plate-incorporation method with bacterial cultures fully grown overnight, as described by Ames et al. (1975). Assays generally were performed with strains TA98 and TA100, obtained from Dr. B.N. Ames (University of California, Berkeley, CA), and with the nitroreductase-deficient strains TA98 NR and TA100 NR, obtained from Dr. H.S. Rosenkranz (New York Medical College, Valhalla, NY). The microsome-containing rat-liver supernatant (S-9) was prepared from Aroclor-1254-induced male Sprague-Dawley rats and was used with the necessary cofactors.

All assays included tests with positive control compounds. The bacterial strains were routinely checked for the presence of known characteristics, including spontaneous reversion frequency, sensitivity to ultraviolet light and crystal violet, and sensitivity or resistance to ampicillin. Benzo(a)pyrene was used as a positive control for the S-9; in TA98, a 5-μg dose of this compound yielded between 300 and 600 revertants/plate with S-9 at 20 and 50 μl/plate.

The sources of the chemicals used were as follows: 2-nitropropane was purchased from Merck-Schuchardt: FRG and 2-nitronaphthalene from EGA-Chemie: and FRG and 1-nitropyrene (labeled 3-nitropyrene) from Koch-Light, England. Di- and tetranitropyrenes were obtained from Dr. R. Mermelstein, Xerox Corporation, USA.

During the course of this investigation, it was found that the strain TA100 could vary in its response to large molecules and that the change in characteristics was not readily detected with crystal violet. The quantitative monitor presently used is 1-nitropyrene (Koch-Light); 1 μg of the commercial product gives between 1000 and

1500 revertants/plate in TA100. Other samples of 1-nitropyrene may give different responses, due to the presence of variable amounts of more mutagenic impurities.

Each sample was assayed with one plate per dose level at two or more dose levels in at least three independent tests. The mutagenic response, expressed as revertants per cubic meter of air for airborne particulate matter and as revertants per gram of consumed fuel for motor vehicle exhaust, was calculated from the linear part of the dose-response curve.

RESULTS AND DISCUSSION

Mutagenicity Pattern of Motor Vehicle Exhaust

Results presented in a previous report (Löfroth, 1979) showed both similarities and differences in the mutagenic responses of *Salmonella* to gasoline exhaust and diesel exhaust from normal passenger cars. All samples were mutagenic in the absence of mammalian metabolic activation, showing that the mutagenic compounds present were either directly acting mutagens or were converted to ultimate mutagens by bacterial metabolism. Both gasoline and diesel exhausts were more mutagenic in TA100 than in TA98.

Enhancement of the mutagenic activity by mammalian metabolic activation was observed for only one type of sample: particulate matter from gasoline exhaust. All other samples were less mutagenic in the presence of S-9 than in its absence. The decrease in mutagenic activity depended on the amount of S-9: it is thus not appropriate to report the mutagenic activity in the presence of S-9 for such samples. For this reason, these results cannot be compared with those of Ohnishi et al. (1980), who reported *Salmonella* mutagenicity data for several gasoline and diesel exhaust samples assayed in the presence of mammalian metabolic activation.

Diesel exhaust was far more mutagenic than was gasoline exhaust. Irrespective of the manner of comparison--revertants per volume of exhaust, revertants per test cycle, or revertants per amount of fuel consumed--diesel exhaust was more than ten times as mutagenic as gasoline exhaust.

Assays involving anaerobic incubation during the first 16 h of the 48-h incubation indicated that the exhaust samples contained no or undetectable amounts of certain nitro compounds that become more mutagenic when assayed under anaerobic conditions.

Mutagenicity Pattern of Urban Particulate Matter

Sampling of urban particulate matter for mutagenicity studies was attempted in the early part of 1976, but the low volumes sampled did not yield conclusive results. High-volume sampling was started at the end of 1977 to test the feasibility of the assay, and regular sampling was begun during 1978.

Several different Soxhlet-extraction solvents--acetone, benzene, cyclohexane, dichloromethane, and methanol--were tested in the early phase of the study. Acetone extraction gave the highest yield of mutagenic activity. Extraction with benzene, cyclohexane, dichloromethane, and methanol followed by further extraction with acetone resulted in samples containing additional mutagenic activity; however, extraction with acetone followed by further extraction with any of the solvents resulted in samples with no additional detectable mutagenicity.

The samples collected above the rooftops were generally tested in the strains TA98 and TA100 and occasionally in the other tester strains, TA1535, TA1537, and TA1538. No detectable mutagenicity was observed with TA1535. Of the plasmid-containing strains TA98 and TA100, the former was usually the most responsive, although TA100 sometimes showed equal or higher responses. Addition of S-9 usually, but not always, decreased the mutagenicity.

It was previously reported (Löfroth, 1979) that many urban particulate samples showed a higher mutagenic activity in the assay involving anaerobic incubation than in the regular aerobic assay. This has been further confirmed, indicating that in contrast to the motor vehicle exhaust samples, urban particulate samples may contain nitro compounds that give this type of mutagenic response.

Seasonal variation. The mutagenic activity of the collected urban particulate matter varied seasonally. Although there were differences between consecutive days (see below), an average mutagenicity could be calculated for periods during which 24-h samples were collected. A number of such periods, consisting of two to four weekdays, were studied. Results to date are presented in Figure 1, which gives the average mutagenic activity in TA98 in the absence of S-9 as a function of the time of year when the samples were collected. The mutagenicity was higher during the winter than during the summer. Many factors--several meteorological parameters as well as emissions of various compounds--may have contributed to the level of mutagenic activity. Thus, it is not certain that the higher mutagenic activity during the winter months was due to heating of buildings.

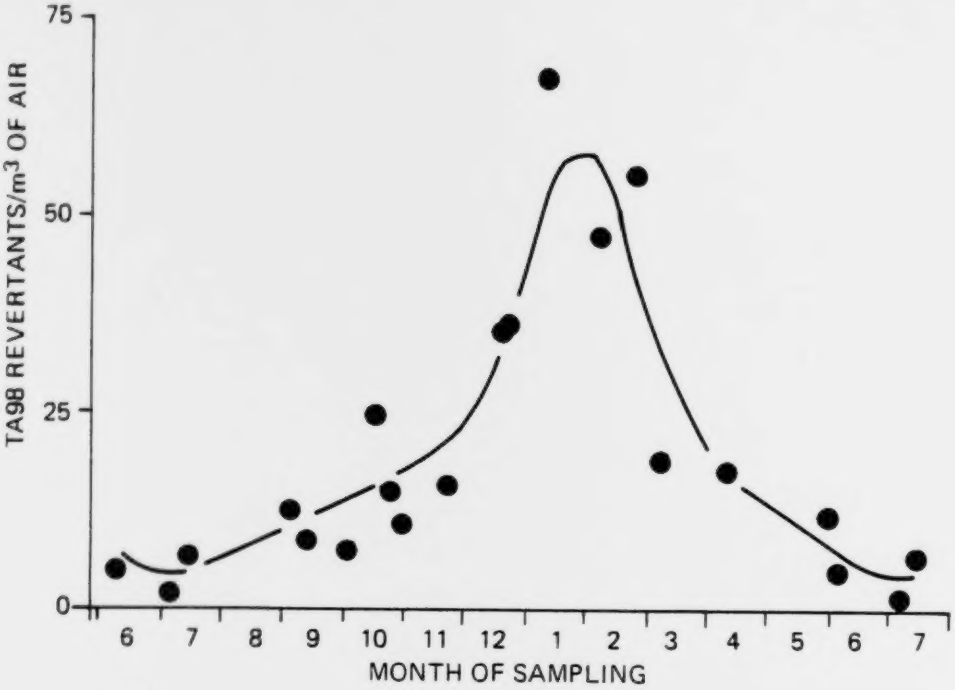

Figure 1. The mutagenicity of extracts of particulate matter collected during weekdays above the rooftops in the inner city of Stockholm. Each point represents the average of two to four consecutive 24-h samples.

Inner city and suburban sites. Mutagenic activity was somewhat higher in particulate matter from the inner city of Stockholm than in that collected simultaneously at the suburban site (Table 1 reports mutagenicity in the absence of S-9). The seasonal variation observed for the inner city sampling site (Figure 1) was also found for the suburban site (Table 1). The suburban site was located in an area where residential heating is generally electric.

Daily variations. The mutagenic activity varied within the same day and between consecutive days. Mutagenic activities in the nighttime hours, when motor vehicle traffic was low, were compared with activities in other periods, in order to assess the contribution from stationary sources such as residential heating. The mutagenicity of particulate matter collected between 10 PM and 6 AM was investigated for three different periods, during which

Table 1. Mutagenic Activity of Extracts of Particulate Matter Collected Above the Rooftops in the Inner City of Stockholm and Simultaneously in a Suburban Area 22 km NNW of Stockholm.

Sampling Period	TA98 Revertants/m^3 of Air[a]	
	Inner City	Suburban
Dec. 20-22, 1978	36	27
Feb. 19-23, 1979	55	30
Apr. 9-12, 1979	18	12
May 28-June 1, 1979	12	7
July 2-6, 1979	2.2	0.9
Sept. 3-7, 1979	13	4.4
Oct. 15-19, 1979	25	13
Dec. 17-21, 1979	35	22
Feb. 4-8, 1980	48	28

[a] Average mutagenicity for two to four consecutive days.

time complete 24-h samples were also collected and assayed. The results from one of these periods are given in Table 2. Nighttime samples were appreciably less mutagenic than the 24-h samples. Similar results were obtained for the other periods: the average nighttime and 24-h samples, were respectively, 5 and 25 revertants/m^3 for Oct. 15 through 19, 1979, and 17 and 48 revertants/m^3 for Feb. 4 through 8, 1980.

These results indicate that emissions during the night, such as those associated with residential heating, were not a major direct source of the mutagenicity present in 24-h samples. However, it cannot be ruled out that some type of enhanced formation of mutagenic components occurred where motor vehicle exhaust was mixed and interacted with emissions from stationary sources.

The variations between consecutive days shown in Table 2 were typical for most of the investigated periods. Changes in meteorological parameters were probably a major reason for these variations. Although the acquired meteorological data have not yet been fully evaluated and compared with the mutagenicity results, a gross comparison revealed that wind speed may have been an important factor in the day-to-day variations in mutagenic activity.

Size distribution. Organic components associated with urban particulate matter for the most part are adsorbed to small, respirable particles (Van Vaeck et al., 1979). Talcott and Harger (1979) have reported that mutagenic activity was associated primarily with particles less than about 2 μm in samples collected in southern California during long-term sampling.

The results for two complete sampling periods of the present project are given in Table 3. It is evident that the smaller the particles, the greater was the mutagenic activity. Also, the mutagenic activity of most fractions decreased after addition of the mammalian metabolic activation system; the exceptions were impactor stage 5 and, in one case, impactor stage 4. Particulate matter collected on these two stages was intensely black, in contrast to the greyish black color of the other fractions.

Samples collected simultaneously without size fractionation had a mutagenicity greater than the sum of the mutagenic activities of the fractionated samples. Reconstitution of the fractionated samples gave about the same mutagenicity as that expected from the sum of the fractions. The lesser mutagenic activity in size-fractionated samples, compared with unfractionated samples, cannot be explained. However, it may have been due to loss of particulate matter to other surfaces of the impactors (Cheng and Yeh, 1979). It is also conceivable that, if mutagens form as artifacts during sampling (Pitts et al., 1978), such reactions may be decreased by

Table 2. Mutagenicity of Extracts of Particulate Matter Collected at Night and Over 24 Hours

Sampling Date (December 1979)	TA98 Revertants/m^3 of Air		
	Inner City		Suburban
	8 h Night[a]	24 h	24 h
17-18	13	48	33
18-19	9	23	12
19-20	12	31	17
20-21	12	36	24
Average	11	35	22

[a] 10 PM to 6 AM.

Table 3. Mutagenic Activity of Extracts of Size-fractionated Particulate Matter Collected in the Inner City of Stockholm Compared with the Mutagenic Activity of Samples Collected Simultaneously Without Size Fractionation[a]

		TA98 Revertants/m^3 of Air			
		Oct. 22-26, 1979		Nov. 19-23, 1979	
Sample	Particle Size (μm)	-S-9	+S-9 (50 μl/plate)	-S-9	+S-9 (50 μl/plate)
Impactor stage 1	7.2	0.3	0.1	0.2	0.2
2	3.0	0.5	0.4	0.3	0.2
3	1.5	0.5	0.5	0.4	0.3
4	1.0	0.7	1.2	0.6	0.5
5	0.5	1.4	2.5	1.0	1.4
Back-up filters		6.2	4.5[b]	5.4	2.7[b]
Sum of impactor stages 1-5 and back-up filters		10	9	8	5
Reconstituted sample: impactor stages 1-5 and back-up filters		-	-	7	7[b]
Filters without impactors		15	9[b]	16	9[b]

[a] Particle sizes are those given by the manufacturer at 50% collection efficiency for spherical particles with unit mass density.

[b] Approximate figures; assays in the presence of S-9 often give nonlinear dose-response curves, making it difficult to arrive at a single response.

collecting precursors on the impactor substrates instead of on the filter through which polluted air is flowing during the entire sampling period.

Chromatographic separation. Extracts of urban airborne particulate matter having sufficiently high mutagenic activity were separated by high performance liquid chromatography (HPLC), and the eluates tested for mutagenicity. The system separates standard polycyclic aromatic hydrocarbons and nitroarenes in narrow peaks (1 ml, 1 or 2 fractions). The mutagenic activity in extracts of particulate matter eluted over a wide range, with no major single peaks (as shown in Figure 2). Most of the mutagenic activity was found in the range where di- to tetracyclic compounds elute. The results suggest that the mutagenicity of extracts of urban airborne particulate matter is caused by many compounds and that it may be unprofitable to look for single compounds as major airborne mutagens.

Mutagenic Response in Nitroreductase-deficient Tester Strains

Salmonella strains deficient in nitroreductase activity were developed in conjunction with mutagenicity studies of nitrofuran derivatives (Rosenkranz and Speck, 1976). Nitrofuran derivatives that are mutagenic in the original strains are less mutagenic in the nitroreductase-deficient strains.

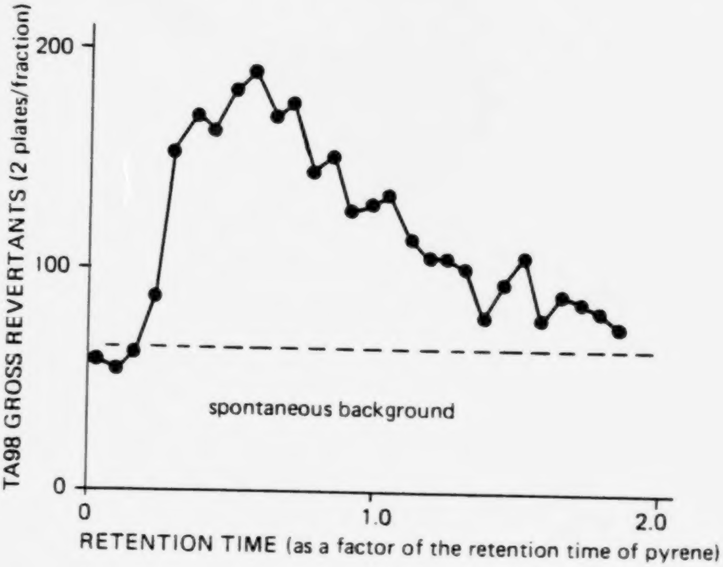

Figure 2. Mutagenic response of fractions from a reverse-phase HPLC separation of an extract of airborne particulate matter. The sample (50 µl, containing activity corresponding to about 1600 revertants) was applied to a Spherisorb S5-ODS column (220 mm x 4.6 mm i.d.) with methanol-water (8:2) as elutant. Fractions of 0.67 ml (20 s) were collected and assayed in TA98 in the absence of S-9, using 0.2 and 0.4 ml.

In the present study, nitroreductase-deficient strains TA98 NR and TA100 NR were used to investigate the behavior of some nitroarenes, motor vehicle exhaust samples, and extracts of airborne urban particulate matter. Typical mutagenic nitroarenes were less mutagenic in the TA98 NR strain than in the original strain (Figure 3); but whereas the mutagenic response of the dicyclic 2-nitronaphthalene almost disappeared, the response decreased only slightly for the tetracyclic 1-nitropyrene. Strains TA100 and TA100 NR behaved similarly.

The mutagenic activity of the motor vehicle exhaust samples was more or less the same in TA98 and TA98 NR (Figures 4 and 5 and Table 4). In contrast, several extracts of urban airborne particulate matter were less mutagenic in the nitroreductase-deficient strain (Figure 6 and Table 4). Preliminary studies comparing the mutagenic activity of these samples in TA100 and TA100 NR gave similar results: the motor vehicle samples produced about the same response in both strains, whereas airborne particulate matter was less mutagenic in TA100 NR than TA100. These results indicate that extracts of urban airborne particulate matter contained certain types of nitro compounds (specifically, those that were metabolized to ultimate mutagens by the bacterial nitroreductase system that had been abolished in the deficient strains). The motor vehicle exhaust samples did not seem to contain detectable amounts of these compounds.

Thus, comparisons between the regular assay and the assay involving anaerobic incubation and between the regular and the nitroreductase-deficient strains both imply that extracts of filter-collected urban airborne particulate matter contain nitro compounds. Due to the uncertainty about artifactual formation of nitro compounds during sampling (Pitts et al., 1978), it is, however, premature to state that airborne particulate matter contains nitro compounds prior to collection. Wang et al. (1980), after comparing the mutagenic response in TA98 and a nitroreductase-deficient strain, suggested that nitroaromatic compounds are present in airborne particulate matter.

Environmental Nitro Compounds

The simultaneous presence of organic compounds and nitrogen oxides in combustion emissions and polluted air requires extensive studies on the possible presence and formation of mutagenic or carcinogenic nitro and nitroso compounds (Pitts et al., 1978; Ehrenberg et al., 1980).

Aliphatic nitro compounds should not be discounted as mutagens. In a series of tested nitroalkanes, the common industrial solvent 2-nitropropane was found to be mutagenic in the

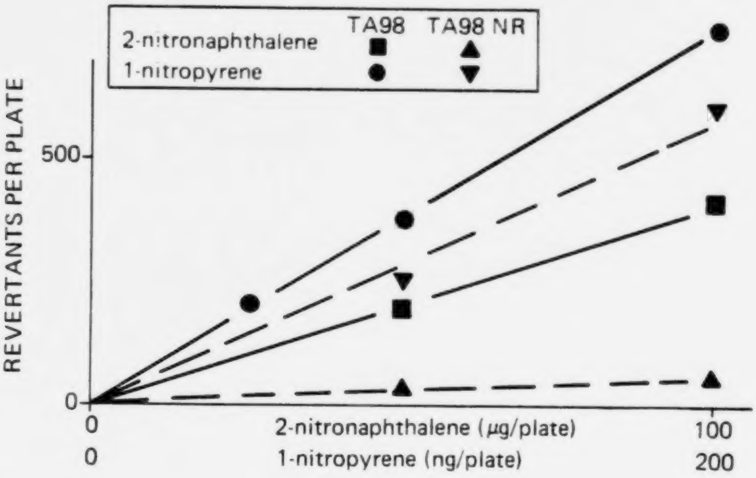

Figure 3. Mutagenicity of 2-nitronaphthalene and 1-nitropyrene in TA98 and TA98 NR in the absence of S-9. The spontaneous mutation rate has been subtracted.

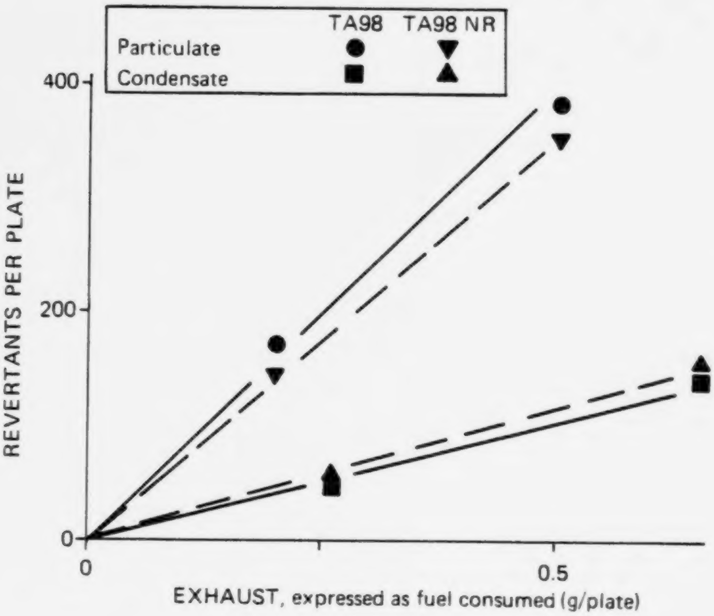

Figure 4. Mutagenicity of diesel exhaust in TA98 and TA98 NR in the absence of S-9.

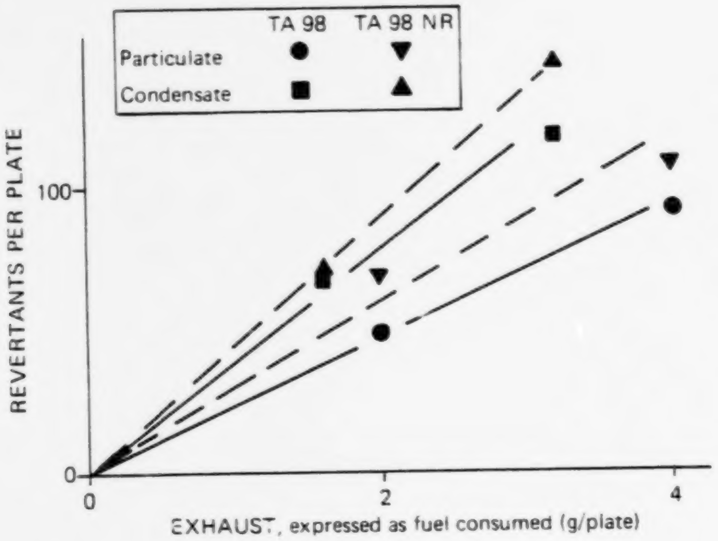

Figure 5. Mutagenicity of gasoline exhaust in TA98 and TA98 NR in the absence of S-9.

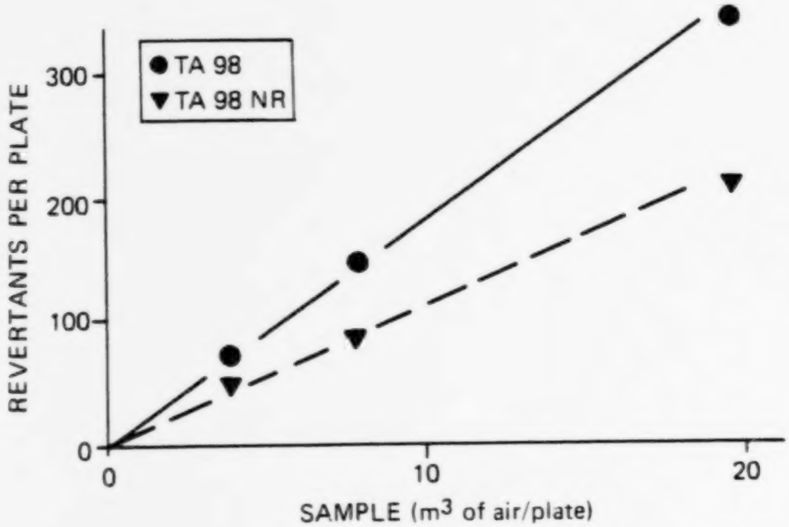

Figure 6. Mutagenicity of urban airborne particulate matter in TA98 and TA98 NR in the absence of S-9.

Table 4. Absolute and Relative Mutagenic Response of Motor Vehicle Exhaust Samples and Extracts of Urban Airborne Particulate Matter in TA98, TA98 NR, and TA100 in the Absence of S-9

Sample	TA98		Percent of Response in TA98	
	Revertants/ g Fuel Used	Revertants/ m^3 of Air	TA98 NR	TA100
Diesel Exhaust				
Particulate matter	800	-	90	>100
Condensate	210	-	110	>100
Gasoline Exhaust				
Particulate matter	24	-	125	>100
Condensate	39	-	115	>100
Urban Airborne Particulate Matter				
4 days: March, 1979 (composite sample)	-	19	70	<100
4 days: Nov., 1979 (composite sample)	-	18	60	<100
4 days: Dec., 1979 (average)	-	35	60	<100

Salmonella assay (unpublished data). The mutagenicity of 2-nitropropane was higher in TA100 than in TA98 and was not related to the mutagenicity of nitrite. The mutagenic behavior of 2-nitropropane suggests that higher homologs of aliphatic nitro compounds may have contributed to the mutagenicity of motor vehicle exhaust samples.

Recent studies show that some photocopies and toners contain mutagenic compounds, and it has been suggested that tri- to pentacyclic nitroarenes were responsible for the mutagenicity in some cases (Löfroth et al., 1980). In one case, the problem was traced to the presence of small amounts of dinitropyrenes present as impurities in a particular carbon black product (Rosenkranz et al., 1980).

Hughes, et al. (1979) reported that pyrene adsorbed to various particulate substrates is nitrated in the presence of nitrogen dioxide and nitric acid and that mononitropyrene can be further nitrated to dinitropyrenes. Nitropyrenes are strongly mutagenic in the Salmonella system and gave the following responses in TA98:

1-nitropyrene	4	revertants/ng
1,3-dinitropyrene	300	"
1,6-dinitropyrene	450	"
1,8-dinitropyrene	750	"
1,3,6,8-tetranitropyrene	60	"

Except for 1-nitropyrene, none of the nitropyrenes showed an increased mutagenic response with anaerobic incubation. In addition, 1,6- and 1,8-dinitropyrene gave about the same mutagenic response in the nitroreductase-deficient strain TA98 NR as in TA98. Nitropyrenes and particularly dinitropyrenes do not act in the same manner as several other nitroaromatic compounds. Thus, dinitropyrenes or similarly behaving mutagenic nitroarenes can be present in motor vehicle exhaust samples and extracts of airborne particulate matter without being detected by the currently used modifications of the Salmonella test.

Model calculation

The investigated urban area--Stockholm and neighboring communities--has an area of approximately 3650 km^2, of which the inner city of Stockholm occupies about 50 km^2. The average fuel consumption for transportation is estimated at 0.7 metric ton/year for the whole area; 0.1 metric ton/year is used in the inner city. Approximately 10% of the fuel is diesel. If the mutagenic activity of vehicle emissions is assigned the values 200 revertants/g gasoline consumed and 2000 revertants/g diesel fuel consumed (Lofroth, 1979), the mixed use then corresponds to an emission equivalent to 400 revertants/g transportation fuel consumed. The average emission per unit area and unit time can then be calculated to be about 100 revertants/m^2/h in the inner city and 8 revertants/m^2/h in the outlying areas.

If the specific emission from an area is known, the resulting concentration in the space above the area can be approximately calculated by a box model:

$$C = \frac{E \cdot x}{L \cdot v} = \frac{1}{L \cdot v} \cdot \sum_{n} E_n \cdot x_n$$

where C = concentration; E = emission/(area)(time); x = distance along the emission area; L = mixing height; and v = wind speed, ≠ 0.

If it is assumed that concentrations are monitored in the center of circular areas, the distance over the inner city is about

4 km and over the outlying areas, about 30 km. The mixing height and the wind speed may be assigned values of 200 m and 3 m/s, respectively. Using these figures, the average mutagenic activity can be calculated as 0.3 revertants/m^3 of air. Actual measurements of mutagenic activity reported in the present study were appreciably higher, e.g., 20 to 50 revertants/m^3 during the winter months.

The difference between calculated and measured average mutagenic activities seemed to be too large to be due solely to the use of a simple model. Several other explanations are more or less probable:

1) The actual mutagenic activity of motor vehicle emissions was 10 to 100 times higher than that from the tested passenger cars. Such levels would not conform with reported data (Löfroth, 1979; Ohnishi et al., 1980).

2) The emissions from DI heavy-duty diesel engines were not considered separately. However, preliminary studies of exhaust samples from such an engine indicate that the mutagenic activity is of the same order of magnitude as that from IDI diesel engines (Rehnberg and Löfroth, unpublished data).

3) There are other major sources of mutagenic components. Analyses of nighttime-collected urban particulate matter indicate that residential heating, etc., was not a major direct source of the mutagenic activity. Currently, most of the stationary energy production in the Stockholm area is from oil combustion. Residential heating is to a large extent provided by larger district plants, hopefully having low emissions of organic compounds.

4) Mutagenic components in motor vehicle exhaust samples are not the same as those in extracts of urban particulate matter. This explanation is supported by the reported differences in the mutagenic characteristics of these samples. These differences and the increased mutagenic activity could be caused by transformations during the residence time in the atmosphere or by artifactual transformations during sampling.

CONCLUSIONS

Several differences in the mutagenic characteristics and in the level of mutagenic activity have been found between motor vehicle exhaust samples and urban particulate matter collected above the rooftops. If major sampling artifacts are absent, the differences imply that transformations occur after emission, changing the composition of mutagenic compounds. It may be that either emissions or actual ambient samples are best suited for analyses.

Much information is still lacking, including evaluations of the adequacy of various sampling methods, mutagenicity studies of samples collected at street level, and mutagenicity studies in conjunction with measurements of other pollutants, including nitrogen oxides. Mutagenicity studies in the Salmonella system can only suggest human health implications, which will ultimately require further evaluation.

ACKNOWLEDGMENTS

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MUTAGENIC EFFECTS OF ENVIRONMENTAL PARTICULATES IN THE CHO/HGPRT SYSTEM

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INTRODUCTION

The emission of particulate matter into the atmosphere from stationary fuel combustion and transportation-related sources is a serious environmental problem. Natusch (1978) has shown that trace elements and chemicals associated with particulate matter from coal combustion may constitute a health hazard. In an earlier study, Natusch and Wallace (1974) reported that many known or potential carcinogens are preferentially concentrated on the surface of respirable coal fly ash. According to Davison et al. (1974), greater quantities of trace elements are associated with particles of fly ash too small to be trapped effectively by conventional particulate-control devices. The potential hazard of respirable particles was brought into sharper focus through studies in which organic extracts of fly ash particles (Chrisp et al., 1978; Fisher et al., 1979) and diesel exhaust particulates (Huisingh et al., 1978) were shown to be mutagenic in Salmonella typhimurium.

Studies with mammalian cells are necessary to confirm the mutagenic effects of environmental particulate matter. The Chinese hamster ovary (CHO) cell is being evaluated as a test system for such particles. These cells, which form discrete cell colonies in culture, were shown by Wininger et al. (1978) to be a convenient system for testing environmental chemicals. In our laboratory we have shown that the CHO cell is capable of phagocytizing particulate matter. This characteristic has been exploited to evaluate the toxicity of a variety of particles (Garrett et al., 1979, 1980), including samples from coal gasification, fluidized bed combustion, and conventional coal combustion. Furthermore, we have shown that the CHO system is useful in determining the toxicity of such diverse environmental agents as liquid effluents from textile mills (Campbell et al., 1979), polychlorinated biphenyls (PCB)(Garrett and Stack, 1980), and organic condensates from a refuse energy recovery system. Because the CHO system is useful in evaluating the toxicity of chemical and particulate matter, the studies have been extended to detect possible mutagenic effects of atmospheric particles. The CHO cell assay, which measures mutation at the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) locus, detects 95% of the chemicals known to have carcinogenic activity *in vivo* (Hsie et al., 1978).

This report examines the use of the CHO/HGPRT system to assay toxic and mutagenic effects of environmental particles. Since CHO cells readily phagocytize whole particles, the system provides a straightforward method for testing mutagenesis of particulate matter without the complexities of extraction and fractionation.

METHODS

The Chinese hamster ovary cell line (CHO-K1) was obtained from the American Type Culture Collection and maintained in medium supplemented with fetal calf serum (screened for virus and mycoplasma). Exponentially growing stock cultures were harvested by washing the flasks once with phosphate-buffered saline and then with 0.25% trypsin. Cells were plated in Ham's F-12 media containing 10% serum and no antibiotics. The cells were usually treated with a three-day exposure to 1 μM aminopterin to prevent the appearance of spontaneous mutants and were subcultured twice before use in a mutation assay.

Mutation induction at the HGPRT locus was measured by the method of O'Neill (1977). Ham's F-12 media containing 5% dialyzed fetal calf serum and antibiotics was used. After trypsinization, 0.5×10^6 cells were transferred to Corning 75-cm^2 flasks and incubated at 37°C. After a 24-h attachment and growth period, the test sample was added to the medium, and the cultures were incubated for 20 h. The exposed cells were then washed three times with saline, and the cells were harvested and counted.

The initial cell survival was determined in experiments in which aliquots of the cell suspension were added to media in 25-cm^2 flasks (300 cells/flask). The flasks were incubated for seven days, and the colonies were fixed and stained with 0.04% crystal violet.

Mutation induction was determined in cells that were subcultured every 48 h. The flasks were washed and trypsinized, and 1×10^6 cells were added to 75-cm^2 flasks. After eight days of culture, cells were plated for selection and post-expression colony survival. Cloning efficiency was determined in experiments in which 200 cells were seeded in hypoxanthine-free media in 25-cm^2 flasks. Colonies of mutant cells were obtained in 75-cm^2 flasks after seeding 2 x 10^5 cells in media without hypoxanthine and with 10 μ_M_ 6-thioguanine. The flasks for cloning efficiency and selection were incubated at 37°C for seven days, and the colonies were then fixed and stained. The number of phenotypic mutants was monitored by changing the media in the flasks after five days of incubation to media without thioguanine but containing hypoxanthine and 1 μ_M_ aminopterin. The flasks were then incubated an additional five days to develop the colonies.

In each experiment, two replicate cultures were formed for the negative and positive controls and each concentration of the test substance. From each replicate, three flasks were derived for measuring initial cell survival, five for selection, three for the post-expression survival, and three for determining mutants resistant to aminopterin.

Phagocytic activity was determined by adding particulate samples to CHO cells cultured in Lab-Tek microslides. Before particles were added, the cells were incubated for a 24-h attachment period at 37°C in a humidified atmosphere of 5% carbon dioxide in air. After a 24-h incubation with the particles, the cells were washed three times with saline, fixed with methanol, and stained with May Grunwald and then Giemsa solutions. The cells were washed with water and then with acetone-xylene solutions.

Phagocytosis was confirmed by preparing electron micrographs of cells exposed to particulate samples in 25-cm^2 flasks. The cells were preincubated 24 h at 37°C. After treatment with the particles for 20 h, the cells were fixed overnight with glutaraldehyde in Millonig's phosphate buffer. After post-fixation with osmium tetroxide, the monolayers were stained _en bloc_ with uranyl acetate in water, dehydrated with ethanol, and embedded. Sections were examined with a transmission electron microscope.

Size analysis was performed using a Coulter Counter TA II after counting 10,000 to 20,000 particles. The instrument was calibrated using a 100-μm aperture and standard 10.05-μm polystyrene latex sphere. Filtered (0.45 μm) physiological saline was used as the electrolyte. Samples of 1 to 2 mg were added to polystyrene test tubes and vortexed for 2 min. Saline (5 ml) was then added, and the tubes were vortexed for 30 sec. Immediately before counting, each tube was vortexed for 10 sec, and 0.1 to 1.0 ml of the sample was added to 100 ml saline. The particles were sized, and population mean diameters for particles $\geqslant$ 2 μm were determined from cumulative plots.

Data reduction and statistical analysis was performed using modified versions of programs written for the Texas Instruments TI-59 calculator (Garrett and Stack, 1980).

RESULTS AND DISCUSSION

The response of the CHO cells to known mutagenic agents was checked using N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and cis-dichlorodiamine platinum-II ($Pt[NH_3]_2Cl_2$). The platinum complex presumably causes base substitution in DNA by miscoding. MNNG, an alkylating agent, is a more potent mutagen in the CHO system. Both compounds increased the mutation frequency with increasing dose. The effect of platinum on mutation frequency in the CHO cells is shown in Figure 1A. The dose response was linear with a correlation coefficient of 0.99. A concentration of 1.5 μg/ml platinum yielded a mean value of 96 mutant colonies/10 flasks in 12 independent experiments. This dose of platinum gave a mutation frequency of $1.16 \pm 0.38 \times 10^{-4}$ and was selected as a positive reference against which to measure the mutagenic effects of environmental particles. Spontaneous mutation in the untreated (negative control) cells gave rise to a mean of 5 mutant colonies/ 10 flasks (N=23) and a mutation frequency of $5.3 \pm 3.8 \times 10^{-6}$.

The range of mutagenic responses obtained for the negative and positive control is shown in Figure 1B. A logarithmic plot normalizes the standard deviation of the data and illustrates the structure of the distributions. Other investigators have transformed CHO mutation data to correct for the nonhomogeneity of variances and non-normal distributions in test cultures and in untreated controls (Irr and Snee, 1979). Figure 1B shows that the mean mutation frequency for the positive reference was approximately 10 times the maximum frequency for the untreated control, facilitating a comparison with weakly mutagenic substances.

The size distribution of particles tested in the mutation assay is shown in Figure 2. Exhaust particles from two diesel engines had a similar particle size distribution (Figure 2, A and B). Brief sonication of one diesel sample apparently dissociated the particulate, so that a larger number of small particles was observed (Figure 2C). Fly ash from oil combustion (Figure 2D) was also a heterogeneous mixture with a size distribution similar to that of the diesel particles. The fly ash from coal combustion tested in these experiments was < 3 μm (Figure 2F) and 2 to 5 μm (Figure 2H). Iron oxide particles (Figure 2E) were similar in size distribution to the coal fly ashes and were used in these experiments as a reference particulate. Silica particles, obtained from a commercial source, were 4 to 9 μm (Figure 2G).

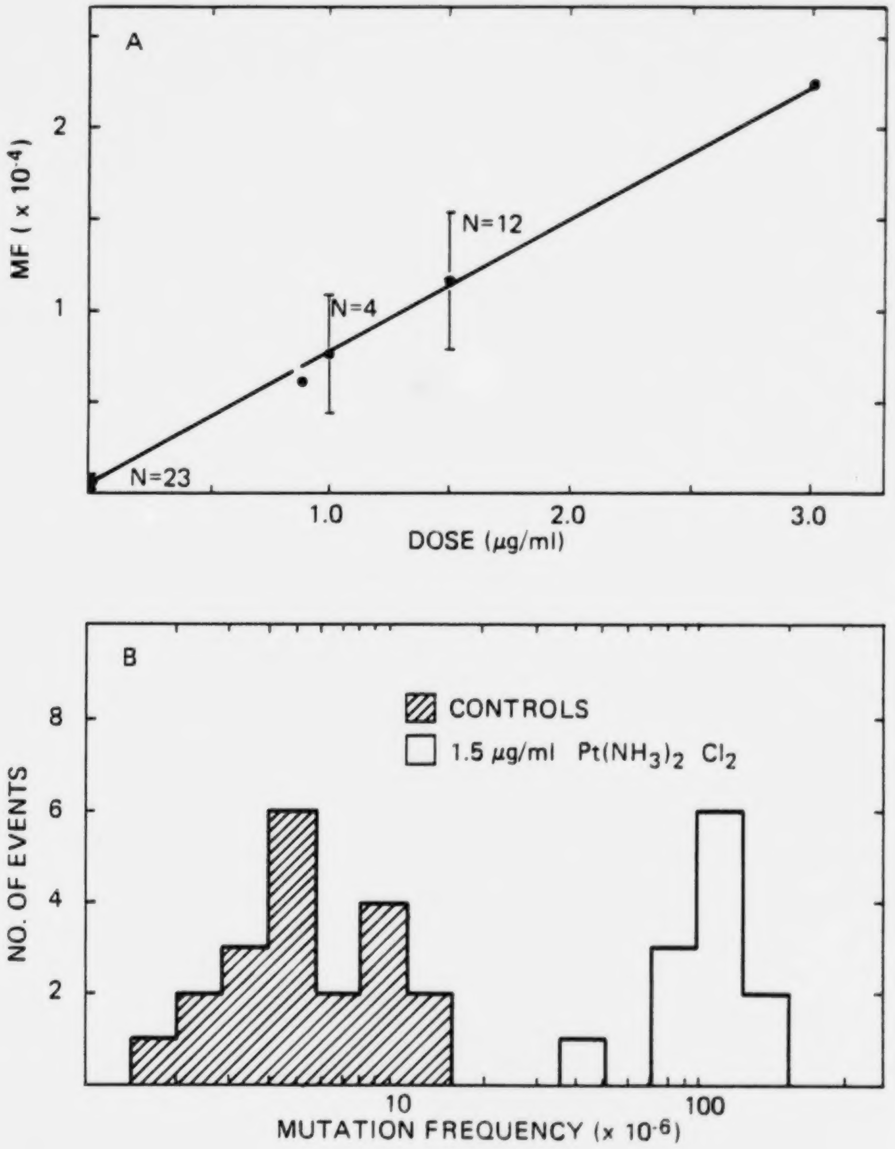

Figure 1. Mutation frequencies (MF) obtained in untreated controls and CHO cells treated with $Pt(NH_3)_2Cl_2$. A. The effect of $Pt(NH_3)_2Cl_2$ on mutation with increased dose (0 to 3 μg/ml). B. Histogram of the mutagenic responses obtained in control CHO cells and cells treated with 1.5 μg/ml of $Pt(NH_3)_2Cl_2$. There were three events with mutation frequencies equal to zero.

The relative size of particles ingested by CHO cells is shown in light and electron micrographs (Figures 3 through 5). Figure 3 shows control cells with very prominent nucleoli and high ratios of nuclear material to cytoplasm; no phagocytic material is seen. Treated cells examined by phase contrast microscopy (not shown) and fixed and stained cells examined by light microscopy revealed a large number of particles in the cytoplasm of CHO cells. These particles were frequently arranged closely around the nucleus. Electron microscopy of sections of fixed and embedded cells confirmed that the particles were in the cells, often closely apposed to the nucleus. The treated cells differed from the control cells in that a membrane could often be seen around each particle or group of particles, forming a very large membrane-limited phagosome. Figure 4 shows CHO cells in which particles of fly ash were present in phagosomes. Frequently the particles either sectioned poorly or appeared to have fallen out of the section. The phagosomes of cells treated with diesel particulates contained small amorphous particles of the diesel exhaust material and empty regions suggesting fluid accumulation (Figure 5).

These experiments provided evidence that a variety of particulates of environmental concern are trapped close to the cell nucleus. The effect of these particles on mutation was investigated by studying both the particles alone and particles in combination with MNNG and $Pt(NH_3)_2Cl_2$. The latter experiments tested the possibility that the particles could facilitate passage of another mutagen to the cell nucleus, with subsequent damage to DNA. Iron oxide, silica, and fly ash particles from coal combustion did not produce a statistically significant difference in mutation frequency in cells treated simultaneously with MNNG and platinum (Figure 6, A and C). Fly ash and silica did contribute to cell toxicity (Figure 6, B and D). The toxic effect of iron oxide with mutagens was not different from that of the mutagen alone.

Because of the high baseline mutation of MNNG and $Pt(NH_3)_2Cl_2$, these experiments would detect relatively large influences on mutation, but not small changes due to the particles alone. Additional experiments tested the effects of the particles without an exogenous mutagen. As shown in Figure 7, A and B, six particles and one organic extract produced mutation in excess of the control values. These substances were toxic to the cell cultures to varying degrees (Figure 6, C and D). The model particles of coal fly ash (2 to 5 μm) produced a small increase in mutation frequency (17×10^{-6} versus 8.4×10^{-6} for controls, at a dose of 125 μg/ml). Another sample of fly ash (0 to 3 μm) from conventional coal combustion caused a four-fold increase in mutation (9.8×10^{-6} at 250 μg/ml versus 2.5×10^{-6} for controls. Similarly, fly ash from oil combustion increased the mutation frequency (29.1×10^{-6} at 75 μg/ml versus 11.8×10^{-6}; $p = 0.15$). Two samples of exhaust

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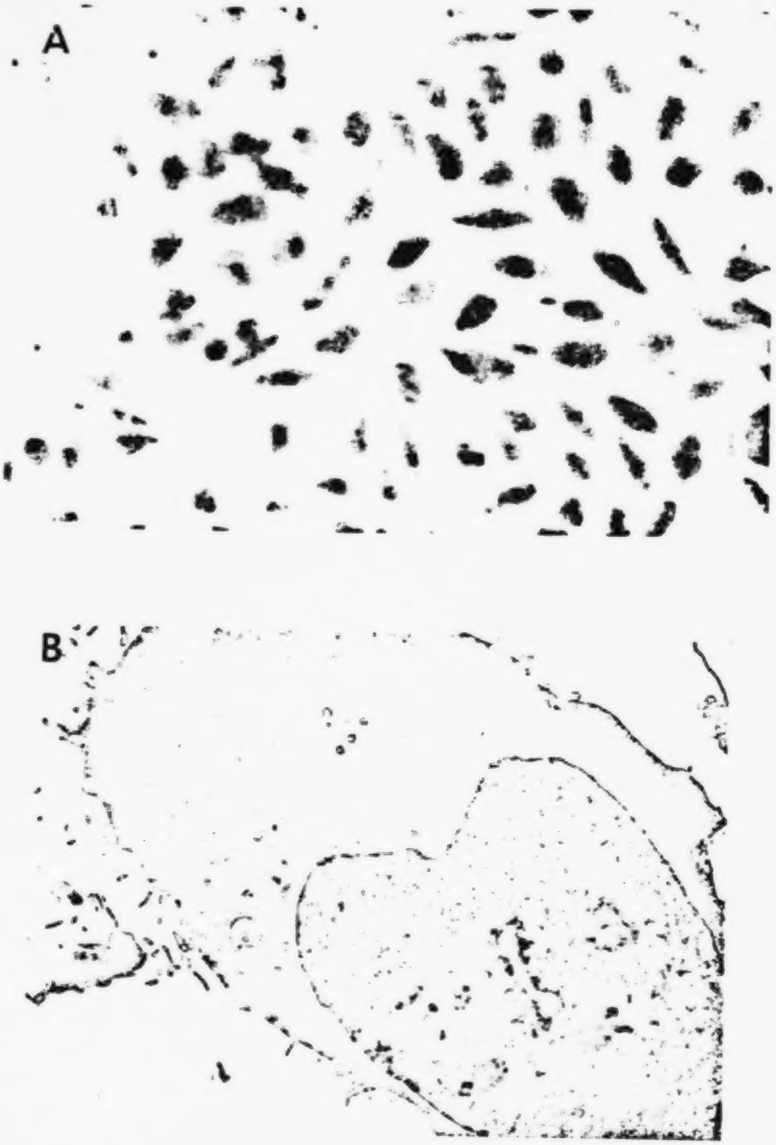

Figure 3. Light micrograph (A) and electron micrograph (B) of control CHO cells. The cells were magnified 200 times in the light micrograph and 5000 times in the electron micrograph. The control cells exhibit prominent nucleoli and high nuclear-to-cytoplasm ratios. No phagocytized material is seen.

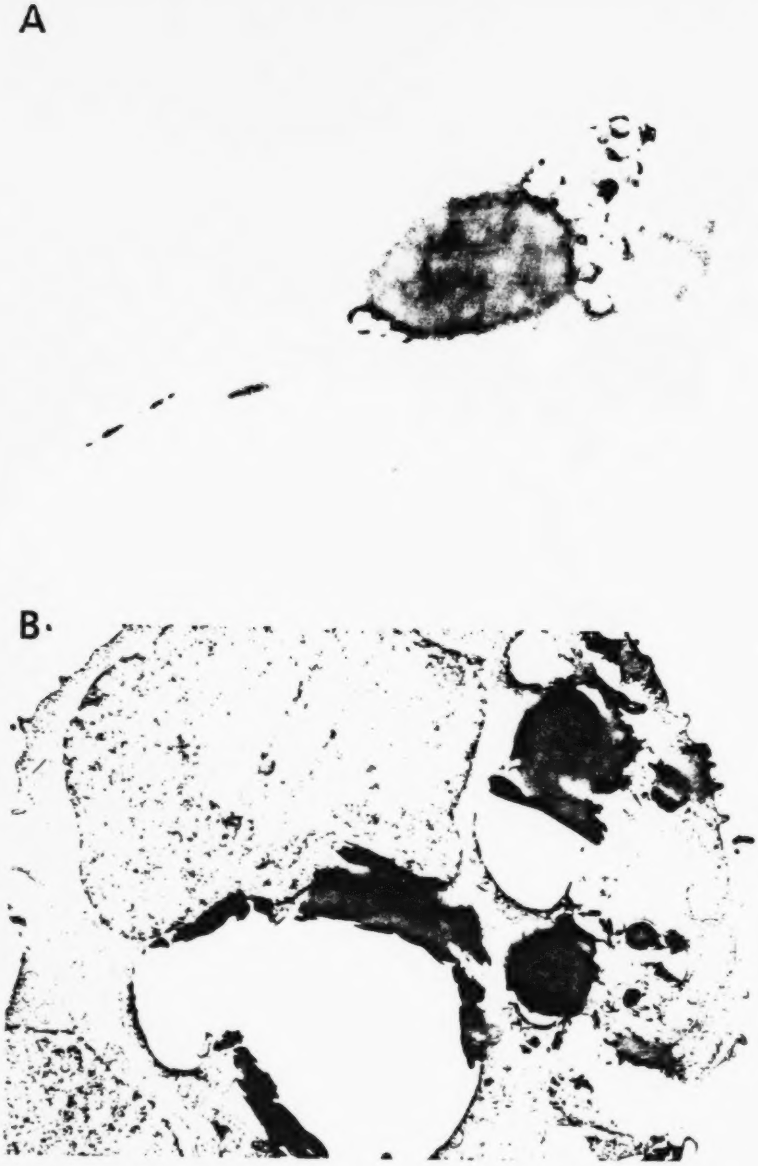

Figure 4. Light micrograph (A) and electron micrograph (B) of CHO cells after a 20-h exposure to coal fly ash. In A, cells were exposed to 200 μg/ml of fly ash, < 3 μm in diameter, and magnified 1000 times; in B, cells were exposed to 250 μg/ml of 2 to 5 μm coal fly ash and magnified 7100 times. Many fly ash particles are visible in the cytoplasm, immediately adjacent to the cell nucleus.

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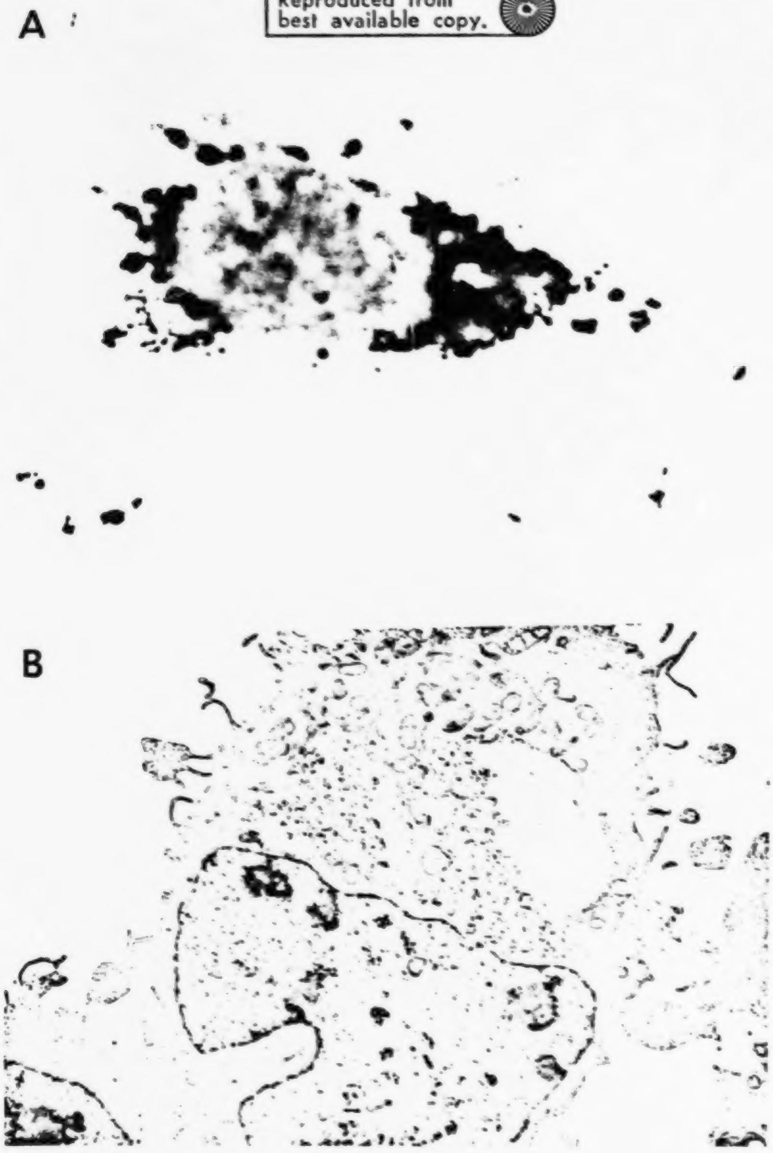

Figure 5. Light micrograph (A) and electron micrograph (B) of CHO cells after a 20-h treatment with sonicated diesel (No. 1) particles at 100 μg/ml. In A, cells were magnified 1000 times, and in B, 5000 times. Phagosomes of the treated cells contain small amorphous particles of the diesel exhaust material. Particles are closely associated with the cell nucleus.

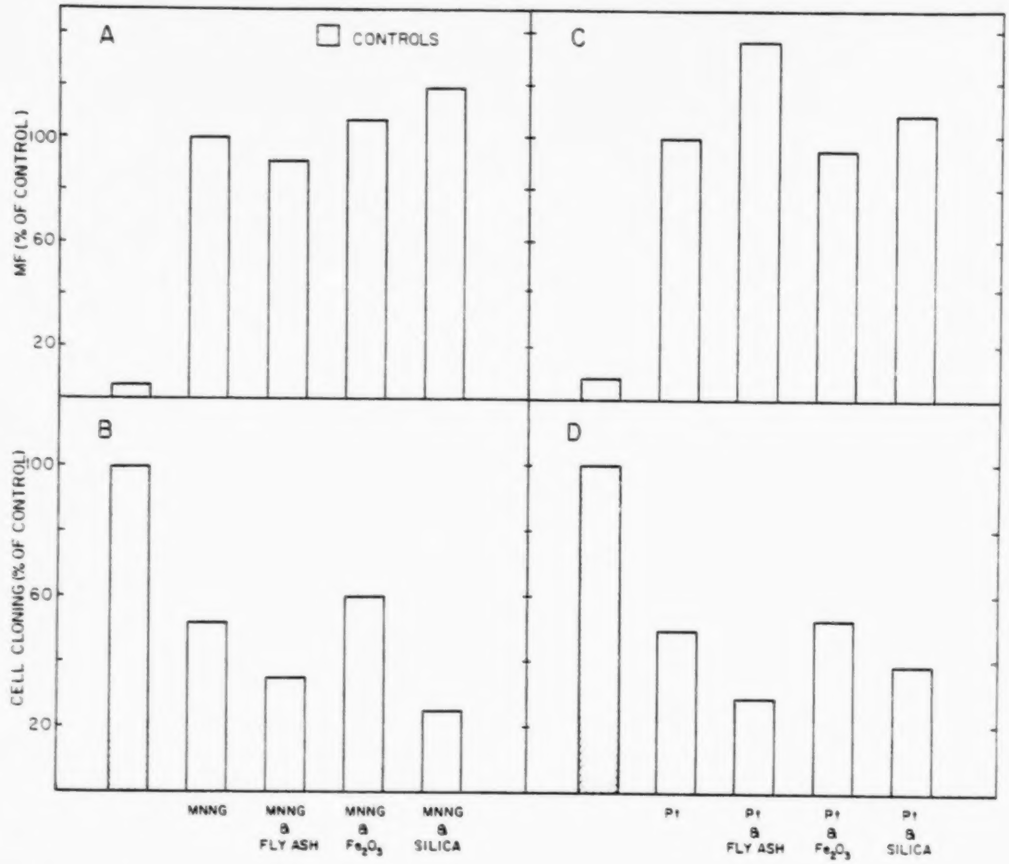

Figure 6. Mutagenicity (A, C) and toxicity (B, D) of particles in combination with MNNG and $Pt(NH_3)_2Cl_2$. Mutagenicity is expressed as a percent of the mutation frequency of MNNG or $Pt(NH_3)_2Cl_2$. CHO cells were exposed to 0 to 3 μm fly ash at 200 μg/ml, ferric oxide particles at 100 μg/ml, and silica particles at 1000 μg/ml. Toxicity was measured as the initial cell survival in the CHO cloning assay. Toxicity data are expressed as a percent of the untreated control.

particles from diesel engines produced about a three-fold increase in mutation over the control values. One sample of particles increased mutation from 2.2×10^{-6} for the control to a value of 5×10^{-6} at 750 μg/ml. A methylene chloride extract of this diesel sample increased mutation to five times the control frequency

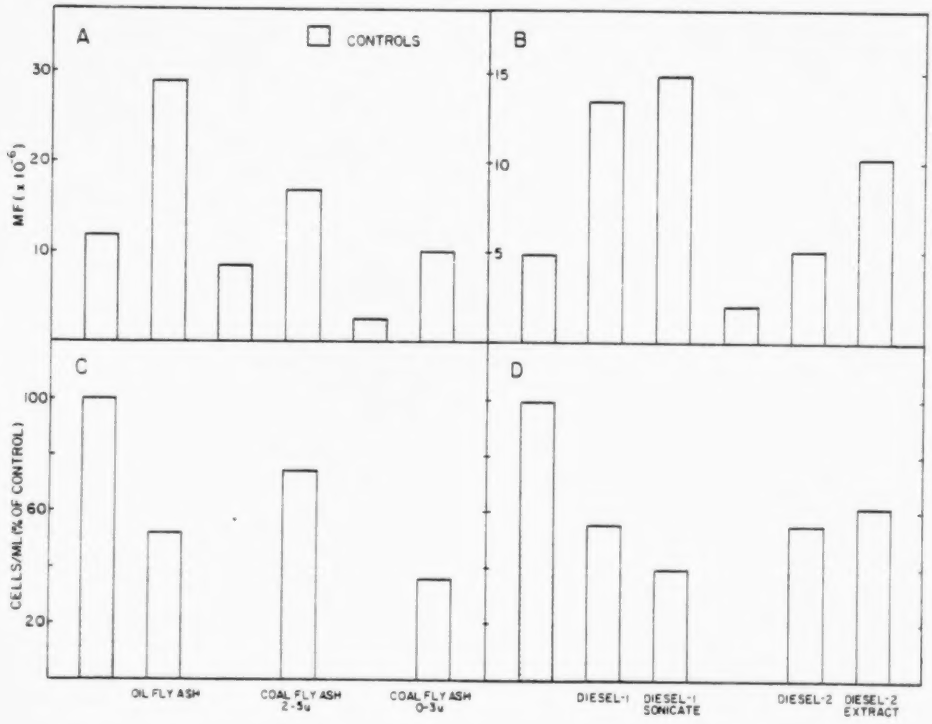

Figure 7. The effect of environmental particulates on mutation frequency (A, B) and toxicity (C, D) in CHO cells. CHO cells were exposed to oil fly ash at 75 µg/ml, 2 to 5 µm coal fly ash at 125 µg/ml, 0 to 3 µm coal fly ash at 250 µg/ml, diesel exhaust particles (No. 1) at 500 µg/ml, sonicated No. 1 diesel particles at 100 µg/ml, diesel exhaust (No. 2) at 750 µg/ml, and a methylene chloride extract of No. 2 diesel particles at 50 µg/ml. Toxicity or cells per milliliter is expressed as percent of the untreated control.

(10.4×10^{-6} at 50 µg/ml versus 2.2×10^{-6} for the control; $p = 0.078$). Higher concentrations of the extract were severely toxic. Another diesel particle was evaluated with and without sonication and mutation was also increased (13.4×10^{-6} at

500 μg/ml for untreated particles ($p = 0.12$); 14.9×10^{-6} at 100 μg/ml for sonicated particles ($p = 0.059$); the control rate was was 4.9×10^{-6}).

These data are, to our knowledge, the first to demonstrate that whole particles of fly ash from coal and oil combustion and exhaust particles from diesel engines can cause mutation in a mammalian cell culture system. For these experiments, it was not necessary to extract the particles with solvents. These results suggest that this test system might be used to assay environmental particulates that have not been previously treated with solvents. Such a test would more accurately reflect natural exposure to particulate matter in the atmosphere, since extraction with organic solvents and subsequent concentration of the extract have no counterparts in natural exposure by inhalation.

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A PRELIMINARY STUDY OF THE CLASTOGENIC EFFECTS OF DIESEL EXHAUST FUMES USING THE *TRADESCANTIA* MICRONUCLEUS BIOASSAY

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INTRODUCTION

Automobile engine emissions are one of the major urban air pollutants in the industrialized nations. The direct and indirect effects of these complex agents on human health are crucial problems for modern society. Epidemiological studies carried out in Europe (Barth and Blacker, 1978; Blumer et al., 1977a, b) and Japan (Shimizu et al., 1977) found that cancer mortality rates were higher in populations near heavily travelled highways than in those away from them. The increasing popularity of diesel-engine-powered vehicles, especially passenger cars, demands a better understanding of the health effects of diesel emissions.

Current information on the health effects of diesel engine exhaust fumes is limited to the results of laboratory tests with experimental animals. In an early review, Goldsmith (1964) concluded that low concentrations of diesel exhaust fumes stimulate the growth of cultured mammalian cells. Studies using mammals such as cats, rats, mice, and Guinea pigs have shown that aryl hydrocarbon hydroxylase increases in the lungs, liver, and prostate; however, no pathological change is seen in the lungs of exposed animals (see Barth and Blacker, 1978, for a review). Campbell et al. (1979), in their studies of the effects of light-duty engine diesel exhaust fumes on mice, found increased susceptibility to infection and mortality rates. Guerrero et al. (1979) found that the sister-chromatid exchange rate in golden hamsters was not altered at a dose of 20 mg/animal for 24 h, while in another study (Pereira et al., 1979) a six-month treatment gave negative results in the bone marrow micronuclei bioassay, but positive results in the sperm morphology test. Other studies have shown biochemical changes in the lungs of rats and Guinea pigs exposed to diesel exhaust fumes. At doses of 250 to 1,500 $\mu g/m^3$, 20 h/day, 5.5 days/week, for 12 to 24 weeks, prostaglandin dehydrogenase activity was reduced (Chaudart and Dutta, 1979). A 36-week exposure caused increases in lung weight and in lipids and collagen in lung tissue (Misioroski et al., 1979). In strain A mice, inhalation of diesel exhaust fumes for seven months showed no effect on lung structure (Orthoefer, 1979), and inhalation for 28 weeks did not cause changes in sperm morphology (Pereira et al., 1979b). Negative results were also obtained in the Drosophila recessive mutation test, after an 8-h exposure to diesel exhaust fumes containing 11.6 ppm of hydrocarbons (Schuler and Niemeier, 1979).

The whole-animal bioassays for diesel exhaust fume effects are time-consuming and costly. An alternative approach to this urgent problem would be using a battery of short-term bioassays similar to those proposed by Huisingh et al. (1979) for diesel exhaust particulates. The Tradescantia (spiderwort) micronucleus (Trad-MCN) is a very sensitive and quick bioassay (Ma, 1980) that is especially suitable for testing gaseous agents in ambient air (Ma et al., 1980), as well as in chambers (Ma et al., 1978; Ma, 1979). The reliability and efficiency of this bioassay were verified by tests of well-known mutagens (Ahmed and Ma, 1980; Ma, 1979; Ma and Anderson, 1979; Ma et al., 1978, 1980a) and dose-response curves established using 1,2-dibromoethane (Ma et al., 1978) and X-ray treatments (Ma et al., 1980b). Therefore, the Trad-MCN bioassay was used to determine the clastogenic effects of total diesel exhaust fumes in the present study.

MATERIALS AND METHODS

The Tradescantia paludosa clone 03 was used for all experiments. A population of this clone was cultivated in the Duke University Phytotron, Durham, NC, under optimal growing conditions in clean air. The plant cuttings bearing young inflorescences (about 5 cm long) were maintained in tap water in a plastic cup (200 ml) before, during, and after treatment. Generally, 15 to 20 cuttings constituted an experimental group, and each experiment involved three groups treated with different doses, one baseline control group (in a clean-air room), and one field control group (in the experimentation area, outside of the treatment chamber). One group exposed to 40 R of X-rays served as the positive control for all experiments.

Two pilot studies were conducted to establish an adequate dose rate and total dose for both the concentration of fumes and the duration of exposure. The final series of experiments was carried out at three different concentrations (1/200, 1/45, and 1/23 dilutions) of the total exhaust fumes. The fumes were generated by a Nissan (1970 Datsun) diesel automobile at the Emissions Measurements Characterization Division, Environmental Sciences Research Laboratory, U.S. Environmental Protection Agency, Research Triangle Park, NC. The automobile ran on a chassis dynamometer under a simulated city driving cycle (23 min/cycle). The diluted exhaust fumes were introduced into a specially designed Plexiglas chamber (31-l capacity) at a flow rate of 32 l/min. Three groups of plant cuttings were each treated with a different dose by exposing them to a given concentration of fumes in the treatment chamber for one, two, or three driving cycles. Gas flow was interrupted for 5 min between the driving cycles. The temperature in the chamber was about 28°C, the relative humidity was in the range of 50 to 80%, and the atmospheric pressure was 0.99 to 1.0 bar (745 to 750 mmHg). Nitrogen oxides, hydrocarbons, carbon monoxide, and carbon dioxide in each of the dilutions of fumes were sampled and analyzed during the treatment. The concentrations of these gases are given in Table 1. The doses of each treatment group were derived from a combination of dilution factor and number of driving cycles.

There was a 30-h recovery time after the end of the treatment, to allow the meiotic prophase I pollen mother cells to arrive at the early tetrad stage, where the broken chromosomes appear as micronuclei (MCN) outside of the nucleus proper. The young inflorescences of treated and control groups were fixed in aceto-alcohol (1:3) after the recovery period; the fixed samples were transferred into 70% ethanol after 24 to 48 h of fixation. Microslides of the tetrad stage of pollen mother cells were prepared by the aceto-carmine squash method. The frequency of MCN on each slide was expressed as the number of MCN per 100 tetrads. An average of 350 tetrads were observed on each slide. The mean frequency and the standard error of the mean for each experimental group was derived from an examination of five slides. The treated and control groups were statistically compared using the standard error of the difference in means, with a significance level of 0.01.

RESULTS AND DISCUSSION

Since the end point of this bioassay is the frequency of MCN in tetrads, and MCN represent the broken pieces of chromosomes, the diesel exhaust fumes effect demonstrated in this study is appropriately referred to as a clastogenic rather than a mutagenic effect. The preliminary data from three series of experiments are shown in Table 1. The only significantly positive results were for DEF-2, T-2, and DEF-3, T-3. All lower doses (fewer driving cycles or lower concentration of fumes or both) gave negative results. The negative result for DEF-2, T-3 was due to an overdose, a common

Table 1. Results of Trad-MCN Test on Clastogenic Effect of Diesel Exhaust Fumes

Experimental Groups (Treatment = No. of Driving Cycles)	Concentration of Gases[a]				MCN per 100 Tetrads (mean)	Standard Error	Significance ($p < 0.01$)
	HC[b] (ppm)	NO (ppm)	CO (ppm)	CO_2 (%)			
DEF-1							
Baseline control					8.32	1.63	
Field control					5.67	0.61	
Treatment 1	3.04	1.5	0.9	0.04	5.45	0.68	–
Treatment 2	3.04	1.5	0.9	0.04	5.97	0.67	–
Treatment 3	3.04	1.5	0.9	0.04	7.75	0.78	–
DEF-2 and -3							
Baseline control					4.76	0.90	
Field control					5.04	0.70	
DEF-2							
Treatment 1	46.95	18.0	18.0	0.49	8.56	2.07	–
Treatment 2	47.36	17.0	16.0	0.49	20.81	3.13	+
Treatment 3	43.84	18.0	17.0	0.49	10.90	1.68	–
DEF-3							
Treatment 1	18.04	8.8	8.0	0.23	7.35	0.32	–
Treatment 2	20.65	10.0	9.5	0.25	7.54	1.62	–
Treatment 3	21.00	9.5	8.5	0.25	14.80	0.44	+
Positive control (40 R X-rays)					62.06	3.10	+

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[a] These concentrations were obtained from the constant volume sampler before a tenfold dilution.

[b] HC = Hydrocarbons.

phenomenon in this test system. Such overdose samples usually have relatively low frequencies of MCN accompanied by abortive cells in tetrads and/or microspores.

The concentrations of fumes that induced MCN were probably comparable to some concentrations measured in a separate *in situ* monitoring project. Samples obtained through 1- to 6-h monitoring at public parking garages, bus stops, and truck stops (Ma et al., 1980a) sometimes gave positive results in the Trad-MCN bioassay. According to Zdrazil and Picha (1978), the particulate exhaust concentration is around 8.21 $\mu g/m^3$ at the orifice of the exhaust pipe, and around 0.205 $\mu g/m^3$ in an ordinary working garage. These figures may help to indicate the actual magnitude of exhaust pollution.

In another study (Ahmed and Ma, 1980), the Trad-MCN bioassay was used concurrently with a human lymphocyte chromosome aberration bioassay to establish the relative effectiveness of these two tests. Results of such comparative studies may be used to extrapolate to human cell systems the mutagenicity of a given agent as determined in the Trad-MCN assay.

The *Tradescantia* stamen hair mutation test (Schairer et al., 1978) is also capable of detecting the mutagenic effect of diesel exhaust fumes. Although no report on direct testing of diesel exhaust fumes in the laboratory is yet available, *Tradescantia* stamen hair *in situ* tests were used at interstate highway junctions and in downtown areas of several large American cities. This kind of *in situ* monitoring is comparable to the present laboratory study. The stamen hair mutation test, which measures the rate of somatic mutation at a particular locus, and the Trad-MCN test, which measures the frequency of chromosome damage, are an ideal combination of bioassays for better assessment of the effect of diesel exhaust fumes on living systems.

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ABILITY OF LIVER HOMOGENATES AND PROTEINS TO REDUCE THE MUTAGENIC EFFECT OF DIESEL EXHAUST PARTICULATES

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INTRODUCTION

An estimate by the U.S. Environmental Protection Agency (EPA) indicates that by 1985, 25% of the automobiles produced in the United States may be diesel powered, because diesel engines provide greater fuel economy than do spark-ignition gasoline engines (Santodonato et al., 1978). Automotive diesel engines may emit from 30 to 80 times more particulates than a comparable gasoline engine (Springer and Baines, 1977). Concern has been expressed about the potential health hazards of these particulates, since extracts of diesel particulates contain chemicals that are mutagenic in the Ames Salmonella typhimurium bioassay (Huisingh et al., 1978; Wei et al., 1980), a short-term test for determining the mutagenic and carcinogenic potential of chemicals (Ames and McCann, 1976).

The majority of the mutagenic activity in extracts of diesel exhausts does not require mammalian liver enzymes for activation. In fact, the addition of rodent liver homogenates (S-9) in the Ames assay mixture decreases mutagenic activity (Huisingh et al., 1978; Wei et al., 1980; Clark and Salmeen, 1980). The mutagenicity-reduction effects of S-9 are also observed when S-9 is added to mutagenic extracts of airborne particulates (Talcott and Wei, 1977) and gasoline-engine exhausts (Wang et al., 1978). Furthermore, S-9 reduces the mutagenic activity of chemicals such as sodium azide, sodium dichromate, sodium nitrite, and 5-nitro-2-furoic acid (deFlora, 1978).

Some investigators have suggested that the mutagenicity-reduction effects of S-9 are due to enzymatic degradation of the mutagen (Clark and Salmeen, 1980; deFlora, 1978): however, no experimental evidence has been brought forth to substantiate this hypothesis. We have examined here the properties and components of S-9 that account for its mutagenicity-reduction effect on diesel exhaust samples. The results indicate that the mutagenicity-reduction effect of S-9 is due to non-enzymatic binding of mutagens to liver proteins.

MATERIALS AND METHODS

The defined diesel exhaust sample used in this study was obtained from General Motors Research Laboratories (GM), Warren, MI. Details on the collection and characterization of the sample have been described by Schreck et al. (1978). The defined sample was collected on a mini baghouse filter attached to a 2.1-l Peugeot diesel engine. The engine was operated on a water-brake dynamometer at a 69-km/m cruise condition and 9 kW (12 hp). Diesel fuel #2 was used. Particulates were weighed, mixed with an appropriate volume of dimethylsulfoxide (DMSO), sonicated for several minutes, and then vortexed immediately before bioassay. The Ames test procedures were followed without modification (Ames et al., 1975). The Salmonella strain employed was TA98, which has been shown to be the most sensitive strain for detecting diesel exhaust mutagens (Huisingh et al., 1978; Wei et al., 1980). The post-mitochondrial supernatant fraction of rat liver homogenates (S-9) was prepared from Aroclor-1254-treated rats, according to the standard methods of Ames et al. (1975). The cofactors were added to the S-9 prior to bioassay. One half milliliter of S-9 mix, containing 50 µl of S-9, was applied on each plate. All experiments have been repeated at least once, and all samples have been tested in duplicate in each experiment.

In some experiments, S-9 was separated according to the procedure of Frantz and Malling (1975). The enzymatic activity in S-9 was inactivated by heat or by omission of the NADPH-generating system. S-9 mix was heated in a boiling water bath (100°C) for five minutes. The boiled S-9 mix was cooled at room temperature and vortexed before being applied to the assay. Part of the heat-treated S-9 was filtered through a 0.45-µm Millipore filter unit to remove coagulated proteins. The filtered or unfiltered heat-treated S-9 mix was then used in the bioassay. The NADPH-generating system was omitted from the S-9 mix by replacement of the cofactor mix with an equivalent volume of sodium phosphate buffer.

Albumin was removed from the cytosol fraction by gel column chromatography. In this experiment, uninduced perfused rat liver nomogenates were used. The column was prepared according to the method of Cuatrecasas (1970). Agarose gel (Sepharose 4B-CL: Pharmacia, Sweden) was activated with cyanogen bromide (approximately 250 mg/ml of settled gel) and the cyanogen-bromide-agarose was stirred overnight at 8 to 10°C in 2 vol of 0.1 M sodium carbonate, pH 9, containing 30 mg/ml ξ-aminocaproic acid. The ξ-aminocaproic-acid-agarose produced was washed, resuspended in two volumes of methanol containing 20 mg/ml L-tryptophan methyl ester and 20 mg/ml dicyclohexylcarbodiimide, and stirred for 4 h at room temperature. The gel was washed with several volumes of methanol prior to resuspension in 50 mM monobasic potassium phosphate. Cytosol fractions were passed through the column, and the filtrates collected were termed the "low-albumin cytosol."

The protein content of S-9, cytosol, and low-albumin cytosol was determined according to a method based on Bradford's Coomassie Brilliant Blue G250 dye-binding assay (Bradford, 1976) (BioRad Protein Assay Kit with a BioRad bovine plasma albumin standard). The albumin content of S-9, cytosol, and low-albumin cytosol was measured with a colorimetric method based on the formation of an intense blue albumin-bromocresol green complex (Doumas and Biggs, 1972). Glutathione was measured according to the method of Ellman (1959), which is specific for thiol groups. Reduced glutathione (Sigma) and bovine serum albumin (Metrix) were obtained from commercial sources. Positive controls in the Ames test were 2-nitrofluorene and 2-aminofluorene (Aldrich).

RESULTS AND DISCUSSION

The addition of S-9 to the incubation mixture of the Ames bioassay reduced the mutagenic activity of the GM defined diesel exhaust sample (see Table 1). The decrease in mutagenic activity was proportional to the amount of S-9 added to the incubation mixture (see Table 2). As shown in Table 3, the mutagenicity-reduction effect was still observed with heat treatment of the S-9 mix or without the NADPH-generating system. The mutagenicity-reduction effect disappeared when the heated S-9 mix was filtered to remove coagulated materials. The above results indicate that enzymatic activity was not responsible for the mutagenicity-reducing ability of S-9. It is evident that proteins, the principal denatured materials in heat-treated S-9, produced the mutagenicity-reduction effect.

Table 1. Mutagenic Activity of the GM Defined Diesel Exhaust Particulate Sample in the Presence or Absence of S-9 Mix[a]

	TA98 Mean Net Revertants/Plate[b]	
Particulates (mg/plate)	Without S-9 Mix	With S-9 Mix[c]
Spontaneous	34	43
0.125	584	446
0.25	890	500
0.5	1138	888
1	1567	1090

[a] For details on the collection of the sample, see Schreck et al., 1978.

[b] The mean number of spontaneous revertants was subtracted.

[c] The concentration of protein in the S-9 mix used was 2.9 mg/plate.

Table 2. Relationship Between the Amount of S-9 Added in the Assay Mixture and the Number of Revertants Induced by the Diesel Exhaust Mutagens[a]

S-9 (µl/0.5 ml S-9 Mix)	TA98 Mean Net Revertants/plate	Protein (mg/plate)	Spontaneous Revertants/ Plate
0	1385	0	23
5	1175	0.3	25
10	1052	0.6	25
20	882	1.2	31
50	770	2.9	33
100	702	5.8	34
150	649	8.7	35

[a] One half milligram GM defined diesel particulates was used in each plate. The protein concentration in the S-9 was 58 mg/ml. One half milliliter S-9 mix was applied on each plate.

Table 3. Effects of S-9 Mix Subjected to Various Treatments on the GM Defined Diesel Exhaust Mutagens[a]

Particulates (mg/plate)	TA98 Mean Net Revertants/Plate			
	Without S-9 Mix	With Boiled S-9 Mix	With Boiled and Filtered S-9 Mix	S-9 Mix Without Cofactors
Spontaneous	29	40	44	44
0.0625	185	126	231	15
0.125	380	197	330	103
0.25	641	392	697	395
0.5	1075	855	1130	854

[a] S-9 mix was inactivated either by heat or by omission of the NADPH-generating cofactor system. Heat-denatured materials, mainly coagulated proteins, were removed from the heat-treated S-9 mix by filtration. The filtrate was also applied on the assay. The protein content of the S-9 mix was 2.9 mg/plate.

Albumin is the principal protein formed in the liver (Rothschild et al., 1972). When albumin is removed from the cytosol fraction by gel filtration, the detoxifying activity is reduced by nearly 90% (see Table 4). Exogenous bovine serum albumin, when added to the Ames bioassay, simulates the mutagenicity-reducing activity of S-9 on diesel exhaust mutagens (see Table 5). These results provide strong evidence that albumin in S-9 or from exogenous sources acts as a nonspecific mutagenicity-reduction agent for diesel exhaust mutagens.

Albumin contains thiol groups that may interact with electrophilic mutagens (Peters and Reed, 1978; Ross, 1962). As glutathione is present in S-9, we investigated the activity of glutathione on the diesel exhaust mutagens. Glutathione and other sulfhydryl compounds have been shown to reduce the mutagenicity of certain mutagens (Rosin and Stich, 1979; Hollstein et al., 1978; Srinivasan and Fugimori, 1979). Table 6 shows that glutathione significantly reduced the mutagenic activity of diesel exhaust at doses of 25 μmol/plate or more, but was ineffective at 0.8 μmol /plate. The amount of endogenous glutathione in S-9, 0.05 μmol /plate (in 50 μl of S-9), was too small to account for the overall mutagenicity-reduction activity of S-9.

Table 4. The Effects of S-9 Mix, Cytosol, and Low-Albumin Cytosol on the Mutagenic Activity of the GM Defined Diesel Exhaust Particulate Sample[a]

	TA98 Mean Net Revertants/Plate			
	Without S-9 Mix	With S-9 Mix[b]	With Cytosol[c]	With Low-Albumin Cytosol
Particulates (mg/plate):				
Spontaneous	27	42	33	30
0.125	461	362	153	480
0.25	781	656	309	744
0.5	1197	945	760	1061
Protein (mg/plate)	0	1.9	7.5	3.7
Albumin (mg/plate)	0	1.4	5.3	0.3

[a] One half milliliter S-9 mix, containing 50 µl S-9, 0.5 ml cytosol, or 0.5 ml low-albumin cytosol, was applied on each plate. The amount of total protein, and more specifically, the amount of albumin used on each plate are given in table.

[b] S-9 was prepared from perfused livers of uninduced male Sprague-Dawley rats.

[c] Cytosol fraction was obtained from the S-9 according to the method of Frantz and Malling (1975).

These results clearly show that the ability of liver homogenates to reduce the mutagenic activity of diesel exhaust samples is due not to enzymatic activity but to an interaction between mutagens and liver albumin. The nature of the interaction is not known. It may be adsorption of mutagens onto proteins by van der Waal forces, a process frequently observed in the binding of drugs to protein. Binding of mutagens to protein may reduce the effective dose of mutagens to bacterial DNA, so that the observed mutagenicity is decreased. Another possible explanation of the decrease would be that protein might block the transport of the mutagens to the DNA.

Table 5. Mutagenic Activity of the GM Defined Diesel Exhaust Particulate Sample in the Absence or Presence of Various Amounts of Bovine Serum Albumin

Particulates (mg/Plate)	TA98 Mean Net Revertants/Plate			
	Without Bovine Serum Albumin	With Bovine Serum Albumin		
		2.5 mg/plate	12.5 mg/plate	20 mg/plate
Spontaneous	24	23	27	20
0.125	318	207	198	148
0.25	579	362	255	170
0.5	1024	736	534	392
1	1278	1146	859	569

Table 6. Mutagenic Activity of the GM Defined Diesel Exhaust Sample in the Absence or Presence of Exogenous Glutathione

Particulates (mg/plate)	TA98 Mean Net Revertants/Plate		
	Without Glutathione	With Glutathione	
		0.8 μmol/plate	25 μmol/plate
Spontaneous	21	20	20
0.125	456	435	262
0.25	712	673	472
0.375	907	835	670
0.5	1062	1055	836

The chemical identification of the direct-acting mutagens is an important objective in safety evaluation of diesel exhausts. When this objective is obtained, the molecular interactions between the mutagens, glutathione, and albumin may be revealed.

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SESSION 5

STATIONARY SOURCES

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PB82-233438

BIOASSAYS OF EFFLUENTS FROM STATIONARY SOURCES: AN OVERVIEW

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Major point sources of pollutants will be with us for a long time. Ever since western civilization began to industrialize and to require high inputs of energy from combustion, pollutants have been emitted in increasing amounts. In 1977, stationary electric power sources were emitting 19.3 million tons of sulfur oxides, 7 million tons of nitrogen oxides, 3.1 million tons of suspended particles, and 0.1 million tons of volatile organic compounds into the atmosphere of the United States (U.S. EPA, 1978). These figures represent about 70% of the sulfur oxides and 40% of the nitrogen oxides emitted each year. Clearly, stationary sources of air pollutants deserve close attention on the basis of the mass of emissions alone. The upward trend in emissions from stationary sources is expected to level off or decrease in some regions of the United States after 1990 but to continue upward in others, in spite of advances in control technologies. New systems of energy conversion and fuel processing, for example, fluidized bed combustion and coal gasification, will introduce new pollution control problems. The industrial growth anticipated in synthetic fuels will increase the need for stringent analysis of the potential hazardous impact of pollutants in the coming years.

In the next few years, we, as a nation, are faced with important energy-related decisions that will have long-term effects on our quality of life. A certain trade-off between abundant, cheap energy and a pleasant, healthy environment seems inevitable. We should make these hard decisions based on the best information available.

To secure this information is the task of the Environmental Assessment Program of the U.S. Environmental Protection Agency

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(EPA). We have learned a lot about the threats of pollution from industrial air, water, and residual waste streams in the last few years. However, we want to do all we can to avoid being surprised by the cumulative effects of effluents from present processes or the introduction of new materials by a shift in technology. The nation's economic growth depends, in large measure, on industrial expansion. To aid industry and regulating agencies in protecting our environment, we must know the chemistry and the potential effects of products, by-products, and wastes. We can and must protect the environment, even while we allow industry to grow.

However, environmental assessment is no easy task. For several years we have been systematically measuring, as precisely as possible, the emissions of many industrial technologies, especially energy-processing technologies. The major components of the waste streams are generally well-characterized, but with the increasing concern over trace-level contaminants, complex mixtures, and their subtle, cumulative effects on the environment, we have been faced with increasingly difficult assessment problems.

At a conference two years ago, Stephen Gage (1978) said, "The emergence of the Environmental Assessment Program as a distinct and important part of EPA's environmental research efforts over the past several years is an excellent example of how the Agency's efforts have turned from primarily research in reaction to known environmental problems to include research which anticipates and tries to avoid future environmental problems. This change of emphasis also predated the rechartering of EPA's position toward toxics."

Some of EPA's best efforts in environmental assessment are in the area of coal utilization. It is obvious that the United States is going to have to use much more coal if our economy is to grow. Continued electricity generation is important, and part of the coal will probably have to be converted to gases and liquids to help meet our varied fuel needs. These processes introduce new compounds and mixtures into the stationary source emission picture. Some of them may present a serious health risk. EPA's Environmental Assessment Program activities have made considerable progress identifying the hazards in the processes, but much remains to be done.

It is extremely difficult to obtain representative samples from process streams that are at high temperature and pressure and that often include highly reactive chemical species. Chemically analyzing complex mixtures of chemicals is another great challenge, even with today's powerful analytical chemistry techniques. Once the emissions are chemically characterized, determination of the relative degree of health or environmental hazard is the next difficult step. This activity is particularly troublesome when cumulative effects, such as those which might result from exposure to carcinogenic, mutagenic, or teratogenic agents, are considered. In this third area--determination of potential health or ecological hazards--bioassays add an important dimension to the process of environmental assessment.

Since 1975, EPA's Industrial Environmental Research Laboratory (IERL), in Research Triangle Park, NC, has been conducting a series of environmental assessment programs designed to

1) systematically characterize the physical, chemical, and biological characteristics of all effluent streams from an energy conversion or industrial process;

2) rank those streams according to hazard potential;

3) identify control technology programs needed to reduce the hazard of those streams; and

4) predict the effects of those streams on the environment, in conjunction with the health and ecological research laboratories of EPA's Office of Research and Development.

Examples of EPA environmental assessment programs currently underway include assessments of coal gasification, fluidized bed combustion, stationary conventional combustion, and coal cleaning and liquefication processes. In performing these environmental assessments, IERL is supporting the regulatory and enforcement offices of EPA by anticipating future control technology needs and developing the data bases needed to support development of standards.

The phased approach to source sampling, chemical analysis, and bioassay has been designed to provide the data needed to evaluate potential environmental impact (Dorsey et al., 1978). This phased approach incorporates the three levels of sampling and analysis shown in Figure 1. This scheme, which relies heavily on bioassays, is successfully meeting the environmental assessment goals listed above.

The phased approach was adopted because it offered potential cost savings over a direct approach in which all streams would be carefully sampled and completely analyzed in one pass. In each level of this approach (see Figure 2), both chemical and biological characterizations of an effluent stream are performed. The chemical characterization provides a quantitative numerical rank of a stream's potential hazard based on an engineering model for potential discharge severity. The bioassay characterization provides a direct measure of a biological response. The dual

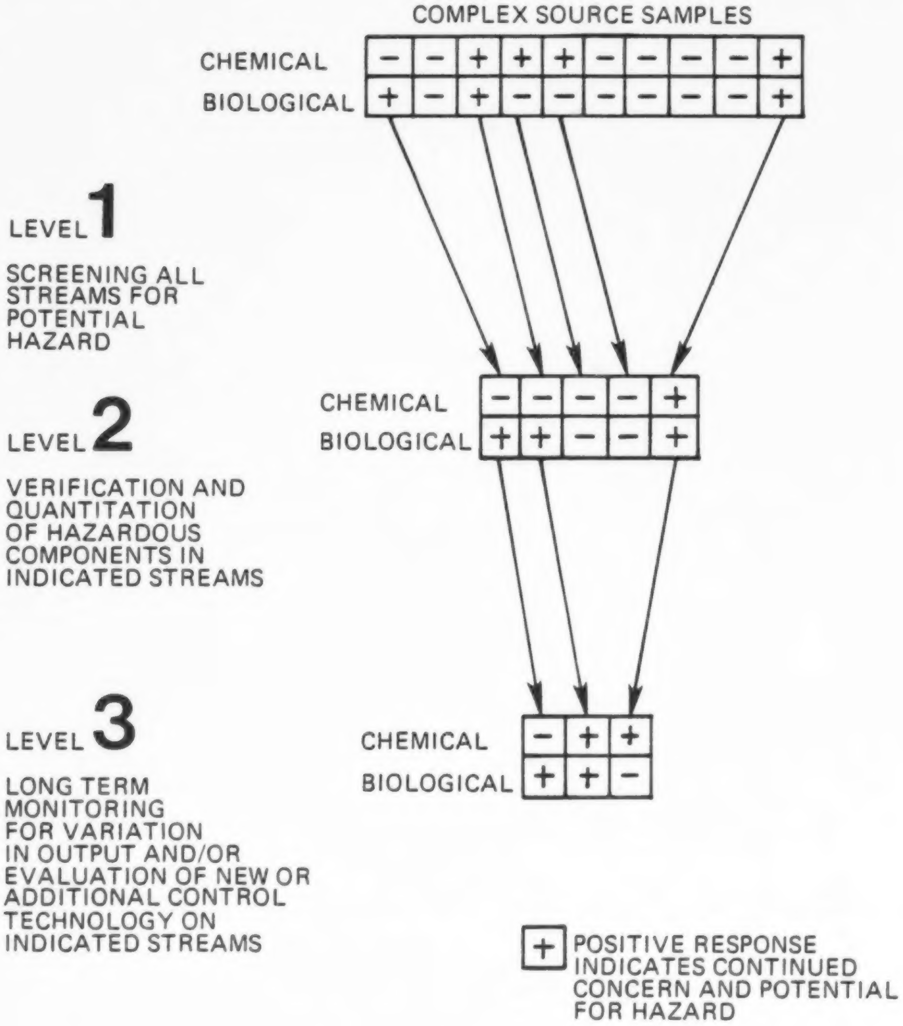
COMPLEX SOURCE SAMPLES
CHEMICAL
BIOLOGICAL
LEVEL 1
SCREENING ALL STREAMS FOR POTENTIAL HAZARD
CHEMICAL
BIOLOGICAL
LEVEL 2
VERIFICATION AND QUANTITATION OF HAZARDOUS COMPONENTS IN INDICATED STREAMS
LEVEL 3
CHEMICAL
BIOLOGICAL
LONG TERM MONITORING FOR VARIATION IN OUTPUT AND/OR EVALUATION OF NEW OR ADDITIONAL CONTROL TECHNOLOGY ON INDICATED STREAMS
+ POSITIVE RESPONSE INDICATES CONTINUED CONCERN AND POTENTIAL FOR HAZARD

Figure 1. The phased approach to environmental assessment.

chemical and biological characterizations are designed to complement each other.

Numerous advantages can be gained by including bioassays in the assessment of stationary sources of pollutants. For example, it may be possible to use organisms the same as or similiar to those likely to be affected by the source. In bioassays, one can frequently use the whole sample without separation or modification,

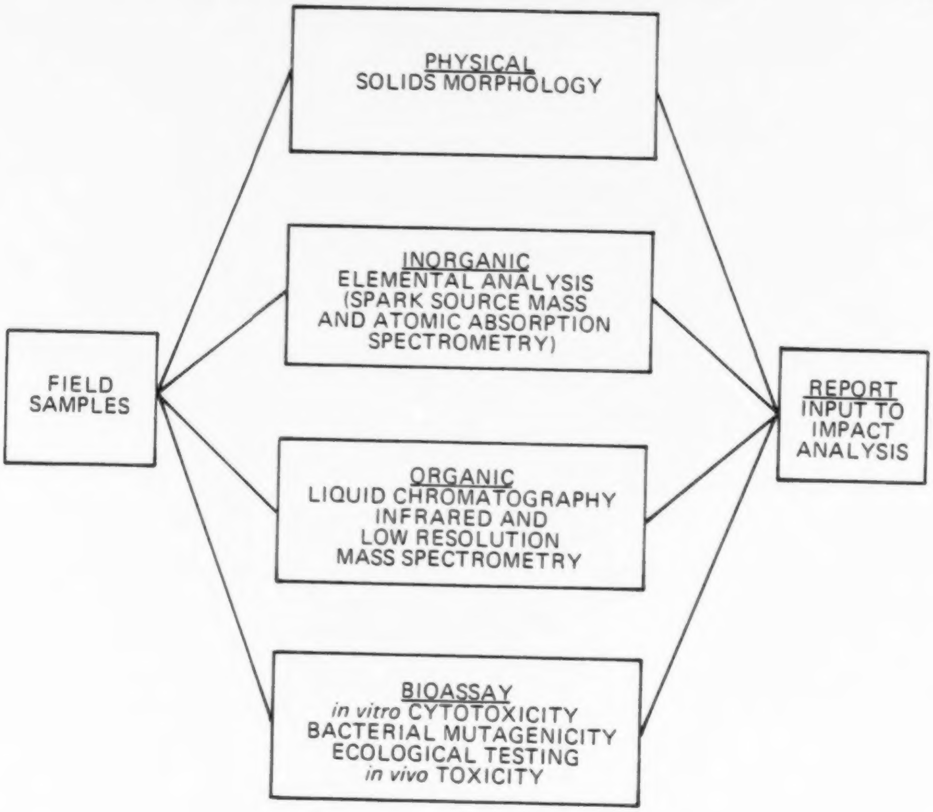
PHYSICAL
SOLIDS MORPHOLOGY
INORGANIC
ELEMENTAL ANALYSIS
(SPARK SOURCE MASS
AND ATOMIC ABSORPTION
SPECTROMETRY)
FIELD
SAMPLES
REPORT
INPUT TO
IMPACT
ANALYSIS
ORGANIC
LIQUID CHROMATOGRAPHY
INFRARED AND
LOW RESOLUTION
MASS SPECTROMETRY
BIOASSAY
in vitro CYTOTOXICITY
BACTERIAL MUTAGENICITY
ECOLOGICAL TESTING
in vivo TOXICITY

Figure 2. Flow chart of Level 1 scheme.

thus allowing the combined synergistic or antagonistic effects to be expressed, which might be either lost in separation or unobserved in chemical analyses alone. We are constantly faced with the dilemma of whether we should separate, extract, and isolate, in an attempt to understand what is present and how it behaves, or work with the whole sample, so that it can express the combined properties. The use of bioassays allows both approaches.

Another advantage is that bioassay results have some built-in interpretation that is absent in chemical analysis. A chemical result indicating the presence of substance X is meaningful only insofar as the potency of X in causing health or environmental damage is known. In contrast, when all the minnows die in an aquatic test, we don't necessarily know why, but it is obvious that this material contains one or more substances likely to be dangerous in aquatic systems.

One disadvantage is apparent in the example above. Bioassays do not always satisfy our desire to know the specific components. Another disadvantage is that of complexity and costs. It is more difficult to set up a laboratory to do routine bioassays at low costs. Bioassays often cannot be started on short notice and thus may require several weeks or months to complete, creating delays and higher costs.

Even though we have argued that bioassays have some built-in interpretative value, they are also sometimes difficult to evaluate. Some often-asked questions are: "Do the results suggest a similar response in other organisms? Can the results be applied to humans? Does mutagenicity relate to carcinogenicity? So what if fruit flies die or don't reproduce?" The bioassays used in the first phase don't provide unequivocal answers. However, they are effective screening devices, providing a basis for subsequent, more definitive tests. All tests must eventually be related to biologic effects. Bioassays, like chemical results, are easier to interpret when supported by other bioassays or analyses.

The best situation we can hope for in environmental assessment is to have a combination of a battery of bioassays and the most complete chemical and physical analyses we can afford. The results of chemistry and bioassays support each other and provide a much more complete picture than either alone. Bioassays should complement chemical assays in such a way that the task of assessing potential environmental impact is possible and practical.

The presentations in this session deal with advances in the application of short-term bioassays to complex mixtures from stationary energy-related sources. In particular, the application of *in vitro* and *in vivo* bioassays to fly ash, fluidized bed combustion effluents, and coal-liquefication material are discussed.

It is important that we look not only at present-day processes, but also at technologies of the future, so that when they are commercially applied, we are not surprised by their effects. It is a distorted perspective to consider only today's problems while allowing tomorrow's to get ahead of us and possibly out of hand. It should be our goal to help avoid any potential environmental problems that may be associated with new combustion processes, synthetic fuel production, or energy-conversion processes.

Bioassays of complex mixtures have already contributed to our understanding of processes and hazards. Engineers, chemists, and biologists have worked together in developing new techniques and in interpreting the results. A series of pilot studies on selected stationary sources have taught us a lot about the combined use of chemical and biological tests in environmental assessment. Experience in these research and development programs has allowed refinements in the bioassays to provide cost-efficient, reliable assessment of environmental impact from a wide variety of source and sample types. It should be reiterated that health, ecological, and industrial laboratories are working together on the collection and interpretation of results. The people involved have a right to be proud of their accomplishments, but we all realize the inadequacies of our techniques and the need to improve them. The work reported at this conference is contributing to our margin of safety and flexibility in future energy production.

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COAL FLY ASH AS A MODEL COMPLEX MIXTURE FOR SHORT-TERM BIOASSAY

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INTRODUCTION

Combustion of coal for electric power generation has increased markedly throughout the past decade and is expected to continue to increase throughout the remainder of this century. Coal combustion produces a variety of biologically active inorganic and organic compounds. Of major concern is the release of oxides of carbon, nitrogen, and sulfur; biologically active trace elements: siliceous primary particulate matter; and organic compounds. Most of the primary particulate matter produced during coal combustion is coal fly ash. Interaction of fly ash particles with inorganic and organic compounds formed during the combustion process produces a unique complex mixture. This mixture may serve as a model for other mixtures resulting from interaction of relatively inert carrier particles with biologically active metals and organic compounds; the bulk of atmospheric particulate matter may serve as a matrix for subsequent interaction of airborne vapors and condensable gases. In this report, we review our studies that indicate the complexity of the physical and chemical properties of coal fly ash and attempt to relate such properties to the biological activity of coal fly ash.

SAMPLE COLLECTION

The fly ash samples described below were collected from a western U.S. power plant burning low-sulfur (0.5%), high-ash (20%) coal (McFarland et al., 1977a). Unless otherwise indicated, the samples were collected and size-fractioned in situ at 95°C downstream of the plant's electrostatic precipitator (ESP). On

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occasion, ESP-collected ash was also studied. Four fractions of stack-collected material with volume median diameters (VMD) of 20, 6.3, 3.2, and 2.2 μm and geometric standard deviations of approximately 1.8 were obtained. Material was size-classified using a specially thermostatically controlled (95°C) system containing two cyclones in series followed by a centripeter with 25 parallel jets. Cyclone-separated material was deposited in a collection hopper, while centripeter particles were collected on fabric filters and removed for hopper deposition by cleaning with reverse air jets operating at one-minute intervals. Thus, in contrast to standard filter collection techniques, collected fly ash samples were not continually exposed to the reactive gases in the flue stream. Such an exposure may lead to changes in the chemical composition and biological activity of collected polynuclear aromatic hydrocarbons (Chrisp and Fisher, in press).

Our samples were collected continuously over a 30-day period. Thus, variations in coal composition, combustion conditions, and other parameters of plant operation that may affect chemical composition should be reflected in these samples. In this regard, Kubitschek and Kirchner (1980) have demonstrated the important effects on mutagenic activity of combustion conditions during start-up and shut-down of a bench-model fluidized-bed combustion system.

PHYSICAL AND CHEMICAL CHARACTERIZATION

Microscopic Studies

In previous studies (Fisher, 1979; Fisher et al., 1976; 1978b), we have described the physical and morphological properties of coal fly ash and have generally found it to be an extremely heterogeneous, complex mixture with a variety of morphological forms. Morphology generally depends both on matrix composition and on exposure conditions during combustion. Upon heating, aluminosilicate inclusions in the coal initially become rounded and then through degassing, become vesicular (Fisher, 1979). Further heating results in the formation of solid spheres, hollow spheres (cenosphere), or sphere-within-sphere structures (plerosphere). Crystals form somewhat later in morphogenesis, with internal (quench) crystals forming rapidly during the transition from liquid to solid phase (Fisher et al., 1976). Such quench crystals have been identified as mullite by Gibbon (1979). While internal crystal formation occurs in milliseconds, surface crystal formation appears to be a much slower process, taking days or months. Surface crystals apparently form through sulfuric acid interaction with metals found on fly ash surfaces.

Previous analysis of individual fly ash particles demonstrated extreme matrix heterogeneity among morphologically similar particles (Pawley and Fisher, 1977). More recently, we have completed a detailed comparison of light-microscopic morphology and individual-particle elemental composition using scanning electron microscopic (SEM) X-ray analysis. Comparative light and electron microscopy is a powerful tool for assessing physical and chemical properties of coal fly ash (Fisher et al., in press). We have demonstrated the presence of relatively pure mineral phases, although the bulk of the ash is amorphous and appears to have the composition of the clay minerals generally associated with coal. Pure quartz, calcium phosphate, titanium dioxide, calcium oxide, iron oxide, and alumina have been observed (T.L. Hayes, E.E. Lai, B.A. Prentice, and G.L. Fisher; University of California at Berkeley; unpublished data; 1979). The degree of pigmentation of spherical particles depends on their iron content. Statistical cluster analysis has confirmed the usefulness of the light-microscopic morphological classifications (Fisher et al., in press). In particular, within each of the specific morphological classes defined by light microscopy, the individual particles generally have very similar elemental compositions. However, the most morphologically amorphous particles tend to be most diverse in elemental composition.

We have also recently completed X-ray diffraction analysis of the crystalline phases within coal fly ash (Hansen et al., MS). These studies show the predominant crystalline species in coal fly ash to be quartz, mullite, and magnetic iron oxides. The highest concentration of crystalline material is found in the coarsest fly ash fractions; we found quartz concentrations of 4%, mullite 8%, and iron oxide 0.6%. The crystalline mineral concentrations decrease with decreasing particle size; in the finest fly ash fraction, quartz was 1.3%, mullite 4%, and magnetic oxides 0.03%. We hypothesize that quartz intrusions within the coal itself produce the silica, whereas mullite and magnetite are formed during the combustion and cooling processes. Mullite is usually associated with a quench crystal phase that occurs during rapid cooling and hence is generally encapsulated within the aluminosilicate matrix of the clay mineral. This does not appear to be the case for quartz or magnetic iron oxides.

As described in our earlier work, surface crystal may form (Fisher et al., 1976; 1978b) as the result of chemical interaction or formation of sulfuric acid on fly ash surfaces, possibly through leaching of minerals and heavy metals from within the fly ash by the surface-associated sulfuric acid. Electron microprobe analysis of the larger crystals in fly ash has identified only calcium and sulfur, thus the majority of crystals appear to be calcium sulfite, present as either gypsum or anhydrite. We postulate, however, that sulfate crystal formation may also increase the biological availability of refractory metal oxides through conversion to the more soluble metal sulfates.

We found (as have many other investigators: see Coles et al., 1979; Ondov et al., 1977) that the concentration of the volatile (at coal-combustion temperatures) trace elements or their oxides are highest in the finest fly ash particles. The elements most enriched in the finest fly ash particles are cadmium, zinc, selenium, arsenic, antimony, tungsten, molybdenum, gallium, lead, vanadium, fluorine, and sulfur. However, the relatively refractory elements uranium, chromium, barium, copper, beryllium, and manganese also are enhanced in the finest fly ash fractions. While it appears that vapor-phase condensation enhances volatile trace elements in fine fly ash particles (Natusch et al., 1974), other mechanisms also may be important in this phenomenon. Filtration studies with neutron-activated coal fly ash indicate that some elements are highly enriched in particles of the size range from 0.2 to 0.4 μm VMD (Fisher et al., 1979). In particular, the elements antimony, arsenic, tungsten, uranium, and chromium have a disproportionately high concentration in particles less than 0.4 μm. It has been hypothesized that homogeneous nucleation and subsequent coagulation of primary particles or the condensation of reaction products enhance submicron particles. Relatively high concentrations of biologically active trace elements in submicron particles may present special technological problems in particulate abatement.

We have compared the distribution of elements in fly ash associated with the aluminosilicate matrix with that of elements either in separate mineral phases or associated with the particulate surface (Hansen and Fisher, 1980). Preferential dissolution with either hydrochloric or hydrofluoric acid indicated that more than 70% of the titanium, sodium, potassium, magnesium, hafnium, thorium, and iron is associated with the aluminosilicate matrix. On the other hand, more than 70% of the volatile elements arsenic, selenium, molybdenum, zinc, cadmium, tungsten, vanadium, uranium, and antimony are associated with the particulate surface. It also appears that the larger portion of the calcium, scandium, strontium, lanthanum, and the rare earth elements is associated with a separate mineral phase, possibly an apatite phase, which has a particle size distribution similar to that of the aluminosilicate phase. These findings, then, allow computation of the probable biological availability of trace elements in coal fly ash. In agreement with these observations, we have found relatively high solubilities for molybdenum, calcium, selenium, barium, arsenic, tungsten, zinc, and antimony in coal fly ash treated with a tris-hydrochloric acid buffer at pH 7.4 (Fisher et al., 1979b).

IMMUNOTOXICOLOGY STUDIES

Although many sensitive *in vitro* bioassays exist for mutagenesis, few are available for screening environmental toxicants for their potential effects on host cellular defense factors. The development of assays for monitoring immunity effects is hampered by the complexity of the cellular and humoral factors involved in the host reaction to neoplasia. We have begun to develop a variety of methods for the study of environmental factors affecting cellular immunities. A major effort has been made toward developing assays that reflect inhibition of macrophage function.

In Vitro Studies

Using SEM X-ray analysis to investigate particles contained in macrophages, we find that the matrix composition of particles contained within phagocytes may vary dramatically (Hayes et al., 1978; Pawley and Fisher, 1977). Furthermore, we hypothesize that variation in the chemical composition of phagocytized particles may also represent variation in the toxicological potential of such particles (Hayes et al., 1978; 1980). We have proposed a model for the exposure of individual lung cells to the foreign elements in fly ash. Segregation of elements in specific particles of fly ash allows much higher exposure levels within individual cells than are predicted by a model based on uniform distribution of elemental concentrations among all particles. Furthermore, we have demonstrated that comparative microscopic techniques can be used to correlate the viability of individual cells with the elemental composition of phagocytized particles. Light-microscopic analysis of macrophages deposited on SEM finder grids and treated with trypan blue dye indicates the viability of the cells: subsequent electron-microscopic analysis of the same cells indicates the elemental composition of the phagocytized particles. Studies are now under way to compare the toxicities of the various fly ash compositions.

To determine whether trace elements in fly ash can alter the function or viability of macrophages, we have calculated the elemental content of macrophages that have phagocytized a few fly ash particles (Hayes et al., 1980). Our calculations indicate that the concentrations of many biologically active trace elements generally are increased by one, two, or even three orders of magnitude. These calculations, then, indicate that the trace elements in fly ash potentially could produce cellular damage. Further studies are necessary to evaluate the biological availability of trace elements in the fly ash. We have found that many trace elements in fly ash inhibit either lectin-induced or mixed-lymphocyte-induced lymphocyte blastogenesis (Shifrine et al., in press). Chromium, lead, vanadium, and copper appear to be effective inhibitors in the lymphocyte-stimulation assays, at concentrations similar to those found in coal fly ash. Interestingly, both lymphocytes and macrophages appear to be extremely sensitive to vanadium.

In vitro exposure of macrophages to particulate matter of similar size distributions allows us to compare the toxicity of fly ash, silica, and glass beads (Fisher et al., 1977: Whaley et al., 1977). Silica was chosen as a positive control because it is a well-documented macrophage toxicant. Glass beads (aluminosilicate particles) were chosen as a negative control because they are apparently inert. The effects of *in vivo* exposure to these particles have been studied using both rat and mouse pulmonary macrophages (Fisher et al., 1977). Particulate exposure was performed at a 40:1 particle:cell ratio, comparable to test particle combinations used in the phagocytic assay (Fisher et al., 1978a). We find that the phagocytic activity of macrophages increases with time in incubation media (Fisher et al., 1977). The degree and rate of enhancement of control phagocytosis depends on the species derivation of macrophages: rat macrophages show the effect after two hours in culture, while murine macrophages take two days in culture. Exposure to fly ash delays the increase in phagocytic rates. The lag can be seen after two and four hours in culture for rat macrophages and after one and two days for murine macrophages. Interestingly, although silica exposure did not delay the increase in phagocytosis, the final phagocytic capability (after seven days) was markedly below that of controls. Further studies are necessary to determine the nature of the enhanced phagocytosis associated with *in vitro* culture and to evaluate the significance of the lag in this effect caused by fly ash.

We have also developed techniques for evaluating the proliferative capacity of lavaged cells from the lung (Boorman et al., 1979a, b). Clonogenic technique was adapted from previously described techniques for quantitation of bone marrow granulocyte-monocyte progenitors (Wilson et al., 1974). The basic culture system involves plating lung-lavaged macrophages into a semisolid methyl cellulose medium. Colonies tend to be smaller and slower to grow than those of hematopoietic cells. Preliminary studies indicate that all particle types (i.e., fly ash, silica, or glass beads) may affect the ability of these lavaged cells to divide (Whaley et al., 1977). Agents that tend to stimulate phagocytosis appear to decrease the cells' proliferative capacity. Similarly, agents that inhibit phagocytosis tend to stimulate proliferation. These observations suggest that particle-induced phagocytic functions may preclude differentiation and subsequent division of progenitors.

We have also demonstrated that cloning techniques are useful in studying the *in vitro* dose-response characteristics of lymphohematopoietic progenitors exposed to trace elements in semisolid culture systems (Wilson et al., 1980). Because of the enhanced concentrations of zinc and selenium in fine fly ash particles and because of the known biological activity of these elements, we evaluated the responses of murine spleen B-lymphocyte progenitors and bone marrow granulocyte-monocyte progenitors to selenite and zinc exposures. At physiological concentrations, both elements significantly suppressed cell proliferation from splenic B-lymphocytes, but not from granulocyte-monocyte progenitors. The results demonstrate the feasibility of using lymphohematopoietic cloning techniques as sensitive short-term bioassays to determine the effects of fossil fuel combustion products on cellular pathways involved in hematopoiesis and immunological processes. We are presently studying the effects of in vivo exposure on the progenitor cell function.

In Vivo Studies

We have also performed in vivo inhalation studies with mice acutely exposed to fly ash and silica aerosols and with rats chemically exposed to fly ash alone. As part of this effort, we have developed techniques for generating well-dispersed fly ash aerosols. We use a Wright dustfeed mechanism for fly ash deagglomeration and aerosolization, a cyclone for separating larger particles, and a krypton-85 discharger for reducing particulate charge to Boltzmann equilibrium (Raabe et al., 1979). In an attempt to improve aerosolization procedures, we compared the efficacy of a fluidized-bed generator to that of the Wright dustfeed system (McFarland et al., 1977b). The results of the study indicate that aerosols produced by the fluidized-bed generator are relatively unstable over time and that deagglomeration is markedly less effective than with the Wright dustfeed mechanism. Aerosols produced by the Wright dustfeeder had smaller aerodynamic size and broader size distributions than those produced by the fluidized bed, with or without use of the Wright dustfeed as a feed mechanism. For these reasons, we have continued to use the Wright dustfeed rather than the fluidized bed for generation of stable deagglomerated aerosols of fly ash, as well as other nonhygroscopic particles.

For acute inhalation studies, we have used exposure via the nose only for up to two hours in small chambers. Chronic inhalation studies have been performed in immersion chambers for periods of up to 20 h per day for 180 days (Raabe et al., 1979). Particle size distributions are continuously monitored using a light-scattering particle counter. We also obtain size-distribution and aerodynamic data using SEM analysis of point-to-plane ESP samples or cascade impactor samples. Total mass is measured periodically in filter samples. For acute inhalation studies, we have used the finest fly ash fractions of the size-classified stack-collected fly ash. However, because chronic inhalation studies require relatively large masses of material, we have employed size-classified material collected from the hopper of the power plant's ESP.

In acute inhalation studies, using a stack-collected fly ash, mice were sacrificed 2, 6, and 15 days after exposure, and macrophage function, pulmonary pathology, and progenitor cell kinetics were evaluated (Fisher and Wilson, 1980). Macrophage functional assays indicated a depression in phagocytic capacity of fly-ash-exposed mice (compared with controls) at 6 and 15 days after exposure. Similar depressed phagocytic activity was observed in silica-exposed animals. Progenitor-cell assays indicated an initial depression in pulmonary alveolar macrophage colonies at 2 days after exposure and a marked elevation at 15 days after exposure. In contrast to the changes observed in pulmonary macrophage progenitors, macrophage precursors in bone marrow and spleen were not significantly affected. These data suggest that the elevated progenitor cell activity is due to recruitment of progenitors from the lung itself (i.e., local production). The increased proliferation of progenitor cells two weeks after acute exposure *in vivo* contrasts with the continued depression observed in *in vitro* studies. Similarly, preliminary studies with intraperitoneal exposure to zinc indicate that lymphohematopoietic progenitors are less sensitive to *in vivo* exposure than are cells exposed *in vitro*.

Further evidence to support the recruitment hypothesis is provided by observing changes in the number of particles within phagocytes (Fisher and Wilson, 1980). For the 2-, 6-, and 15-day observation periods, the number of particles continually decreased within the cells, suggesting enhanced production of phagocytic cells in the lung. On the other hand, we have not observed similar effects with long-term low-level chronic exposure to fly-ash aerosols derived from the power plant's ESP. It is not clear whether the difference in biological response reflects the difference in concentration (200 vs. 2 mg/m^3), the difference in species (rat vs. mouse), or perhaps, most importantly, the difference in fly ash source (stack vs. ESP hopper-collected ash).

We have also developed techniques for quantifying fly ash deposited in rat lungs during the chronic inhalation studies (Fisher et al., 1980). Because it is difficult to separate particulate matter from lung tissue, we chose to evaluate elemental analysis as a measure of fly ash in the lung. Selection of the appropriate element was based on the following criteria:

1) the elemental analysis should be specific, and detection limits should be appropriate for a sensitive indicator of of lung burden;

2) the levels of the element in the tissue should be low and relatively constant;

3) the dissolution of the element should parallel the particulate mass dissolution; and

4) the element should be uniformly distributed throughout the size range of the fly ash under study.

Only aluminum and silicon met these criteria: aluminum was chosen because its analysis is more sensitive and less troublesome than that of silicon. The lung content of fly ash calculated from the aluminum analysis was in quantitative agreement with calculations based on available deposition and clearance data.

We have evaluated the mutagenic properties of coal fly ash extracts using the Ames Salmonella assay system (Ames et al., 1975: Chrisp et al., 1978; Fisher et al., 1979a). Our studies indicate that in keeping with a model of surface deposition, the finest fly ash fractions are indeed the most mutagenic (Fisher, 1980). However, the 3.2-μm fraction of fly ash is more mutagenic than the 2.2-μm fraction. At first we assumed that this was due to antimutagens in the fly ash, and we evaluated the possible role of selenium, as the selenite, or fluorine, as the fluoride. These elements were chosen because of their relatively higher concentrations in the finest fly ash fraction than in the 3.2-μm fraction, and because selenium, as the selenite, is an antimutagen for acetylaminofluorene and its derivatives. Fluoride is generally recognized as an enzyme inhibitor. Adding these elements to extracts of the 3.2-μm fraction, however, did not alter its mutagenic activity. Thus, we do not have experimental support for the hypothesis that antimutagens are present in the finest fly ash fraction.

Natusch (Colorado State University, personal communication, 1980) suggests that the difference in mutagenic activity may be due to differences in chemical absorption of mutagens by the two fly ash fractions. Further evidence for the chemisorption of mutagens on fly ash surfaces is the photostability of these compounds. We have irradiated fly ash samples with ultraviolet light, sunlight, and X-irradiation, with no decrease in mutagenic activity. However, heating the fly ash to temperatures of 200 to 250°C results in the loss of approximately half of the mutagenic activity, while heating above 300°C results in complete loss of detectable mutagenic activity (Fisher et al., 1979a). The biphasic nature of the loss in mutagenic activity with heating indicates the presence of at least two mutagens or classes of mutagens. Most recently, we have demonstrated that the loss of mutagenicity is due to decomposition of surface-associated materials, as opposed to volatilization (Hansen et al., MS), further supporting the suggestion that mutagens are on fly ash surfaces.

Fly ash collected by the power plant's ESP does not appear to be mutagenic in the Ames test (Fisher et al., 1979a). Furthermore, even when classified by size and sampled in a size distribution equivalent to that of our finest stack-collected fraction, ESP-collected materials are still not mutagenic. Our studies indicate that temperatures of approximately 100°C may be critical for absorption of mutagens on fly ash surfaces. Indeed, the calculations of Natusch and Tomkins (1978) indicate that the deposition of vapor-phase polynuclear aromatic hydrocarbons on fly ash particles is extremely sensitive to temperature changes around 100°C.

We have compared the efficiencies of mutagen extraction by a variety of solvents. Normal saline is a very poor extractant of mutagenic activity, whereas horse serum and serum from other species are fairly efficient extractants of fly ash mutagens (Chrisp et al., 1978). We found that a mutagen-serum protein complex forms that can be isolated from the serum, although it is available to the bacterial genome. Further studies indicated that albumen alone is nearly as efficient as the total serum in extracting mutagens from fly ash (C.E. Chrisp and G.L. Fisher, unpublished data). Direct extraction of fly ash with dimethylsulfoxide and sonication results in the highest detectable levels of mutagens.

We have not identified the chemical composition of the fly ash mutagens, although recent studies using acidic, neutral, and basic aqueous fly ash extracts further indicate that mutagens in fly ash are not inorganic. Acidic aqueous fractions were not mutagenic, whereas basic aqueous fractions contained approximately half of the mutagens extractable with dimethylsulfoxide. These results indicated that a significant portion of the mutagenic activity in coal fly ash could be accounted for by the presence of weak organic acids (Hansen et al., 1980).

To evaluate the carcinogenic potential of coal fly ash, we have modified the tracheal implant system described by Griesemer et al. (1974). For these studies, we have packaged fly ash in 0.2-μm Nuclepore filters. This method allows for the slow release of mutagens to the sensitive tracheal epithelial cells and minimizes the risks of local tissue damage and toxicity. We have used the Ames bacterial mutagenesis system to monitor the release of mutagens from fly ash in tracheal implants (Chrisp and Fisher, 1980). Studies are now in progress to evaluate the carcinogenic potential of the fly ash using the tracheal implant system.

CONCLUSIONS

In conclusion, our results demonstrate the extreme complexity of coal fly ash in terms of matrix composition, morphological appearance, and surface trace element and organic chemical composition. Assays are being developed to measure the potential immunotoxicity of fly ash. Acute inhalation studies have demonstrated that coal fly ash may be as toxic as quartz to the pulmonary alveolar macrophage. Further *in vivo* studies comparing the cytotoxicity of fly ash and α-quartz are required to substantiate this hypothesis. The feasibility of applying sophisticated cloning techniques to the evaluation of potential lymphohematopoetic effects from complex mixtures has been demonstrated. Mutagens in coal fly ash appear to be absorbed to fly ash surfaces and hence may exist in the environment for relatively long periods of time. Techniques are now being developed to evaluate the carcinogenic potential of coal fly ash through a combination of bacterial mutagenesis assays and tracheal implant carcinogenesis assays. Detailed chemical, morphological, and toxicological analyses indicate the usefulness of coal fly ash as a model complex mixture.

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POSSIBLE EFFECTS OF COLLECTION METHODS AND SAMPLE PREPARATION ON LEVEL 1 HEALTH EFFECTS TESTING OF COMPLEX MIXTURES

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INTRODUCTION

The Level 1 Environmental Assessment Program of the U.S. Environmental Protection Agency (EPA) Industrial Environmental Research Laboratory (IERL) has two main goals: first, to detect potentially hazardous emissions from stationary sources, and second, to develop a data base that will permit a relative ranking of industrial streams with respect to their potential biohazard. The level of bioassay applied does not permit either qualitative or quantitative risk assessment. However, since both chemical and biological effects are evaluated, the level of data integration and coordination is increased, reducing the chance that a potentially hazardous stream will go undetected.

During the initial phases of the IERL Environmental Assessment Program, considerable time was given to methods of sample collection, preparation, and analysis for chemistry assessment (Lentzen et al., 1978). The recent introduction of bioassays requires a similar appraisal of the functions of sample collection, storage, and pretest handling as they relate to the specific health effects and ecological tests proposed for Level 1 biological assessment.

Because of the need to rank streams according to potential biohazard, the initial approach taken by IERL was to evaluate samples in the specific bioassays in a state as similar as possible to that found at the time of sampling. However, recent results reported in the scientific literature on analysis of complex environmental mixtures have shown that pretest processing of samples (concentration of liquids, extraction of particulate) often

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results in enhanced biological activity (Klekowski and Levin, 1979: Kubitschek and Venta, 1979; Löfroth, 1978: Pitts et al., 1977: Teraniski et al., 1978). In this report, several of the sampling and pretest procedures are reviewed to illustrate how the application of these techniques to Level 1 Environmental Assessment will affect the test responses and ultimately the goals of this program.

METHODOLOGY

The IERL Level 1 Environmental Assessment Program evaluates source emissions according to the following five parameters:

1) rate of release into the environment,

2) distribution between physical states,

3) chemical composition,

4) detection of potential specific health effects, and

5) detection of potential impact on the ecosystem.

A schematic of the above parameters is given in Figure 1.

The initial Level 1 methods manual for biological testing contained protocols for five health effects tests and eight biological tests (Duke et al., 1977). The types of data obtained from these tests were varied and not amenable to interpretation by anyone other than a biologist. Clearly, a method of developing uniform data was needed. Out of a review of Level 1 bioassays and data from several pilot studies, a system of uniform data analysis and formatting was recommended (Brusick, 1980). The review study also recommended elimination or substitution of certain test procedures. Table 1 identifies the current status of recommendations for Level 1 bioassays. No procedures have yet been approved for the Soil Microorganism Toxicity Assay: test substitutions or protocol modifications are being considered for some of the other tests as well.

The data of three pilot studies have been evaluated following the recently proposed data-formulating procedures (Brusick, 1980). The results were recorded as high (H), moderate (M), low (L), or nondetectable (ND) on a summary sheet such as in Figure 2. Table 2 defines the limits of each category.

The studies on Coal Gasification, Fluidized Bed Combustion, and Textile Plant Liquid Effluents received very little pretest sampling or sample history evaluation. Most of the samples were

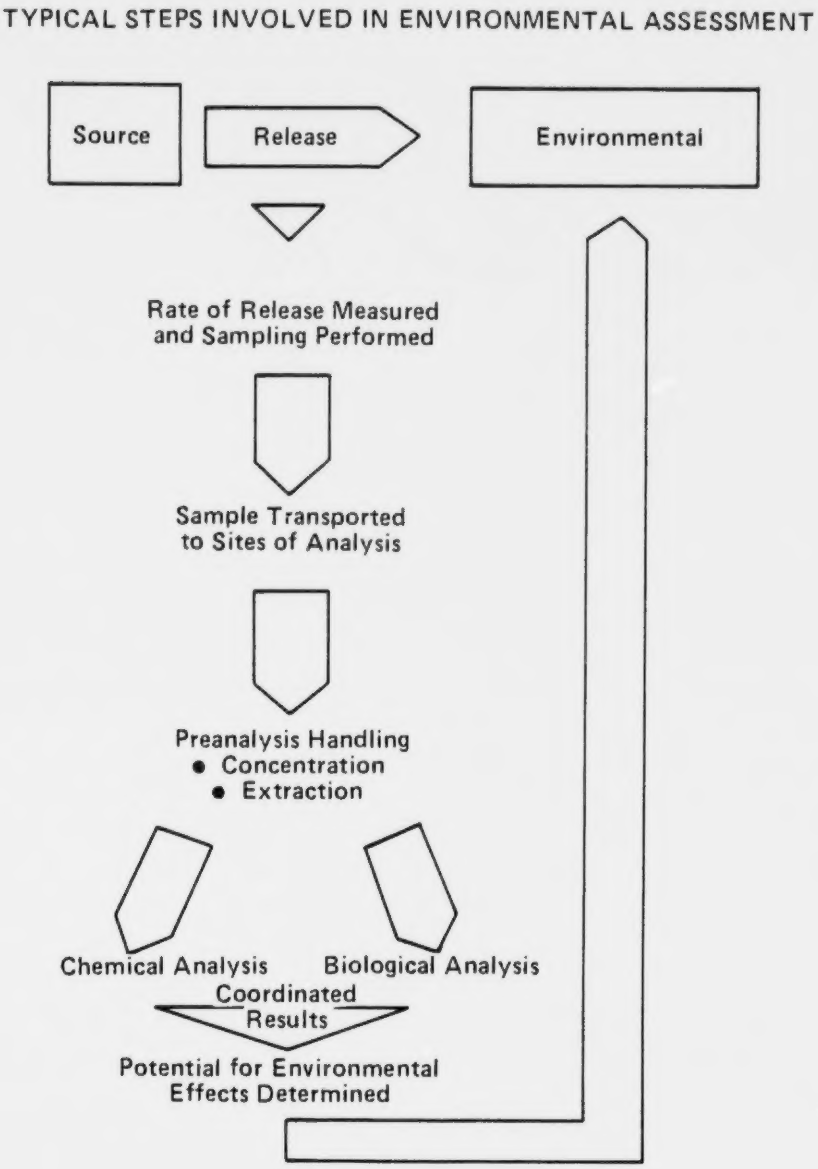
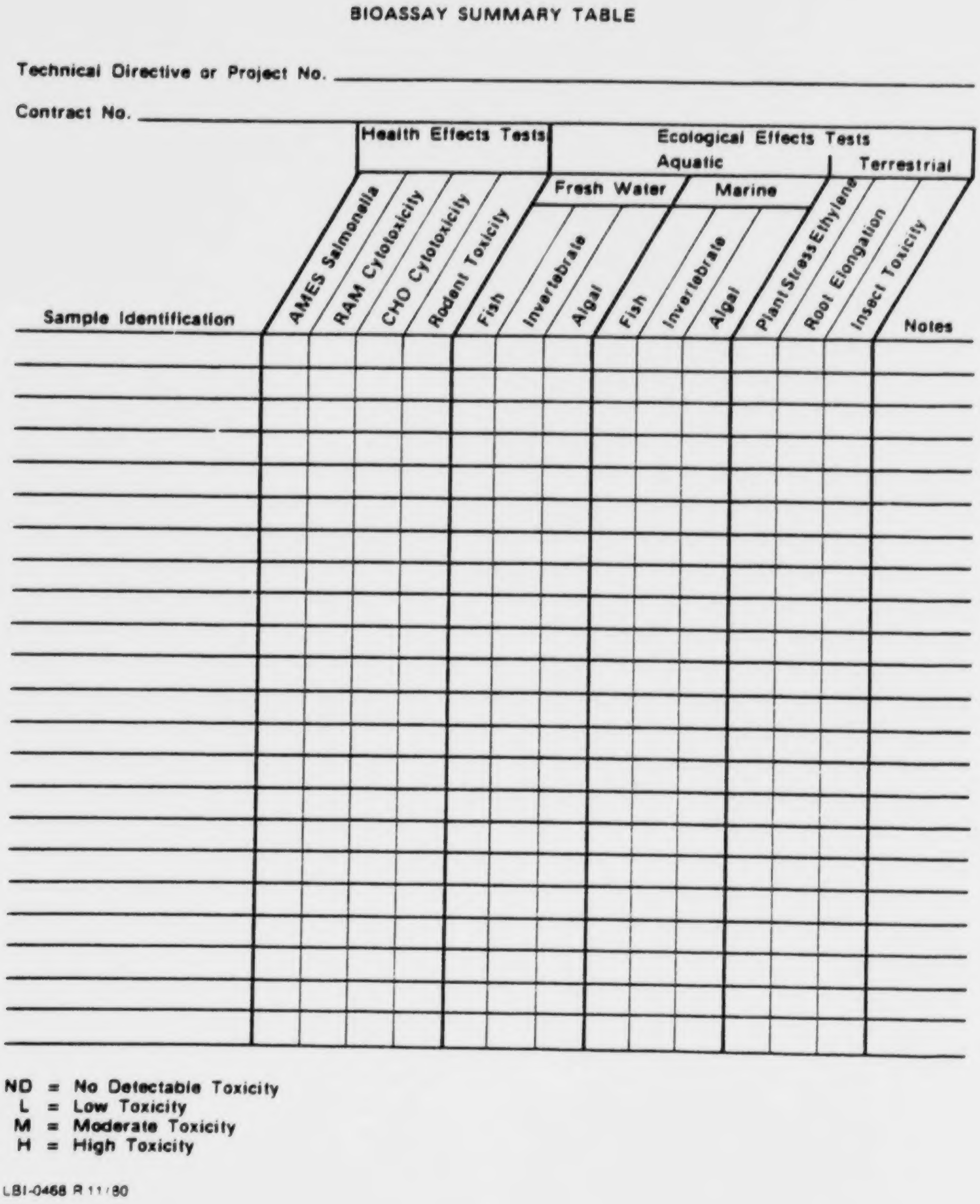

TYPICAL STEPS INVOLVED IN ENVIRONMENTAL ASSESSMENT
Source
Release
Environmental
Rate of Release Measured and Sampling Performed
Sample Transported to Sites of Analysis
Preanalysis Handling
• Concentration
• Extraction
Chemical Analysis
Biological Analysis
Coordinated Results
Potential for Environmental Effects Determined

Figure 1. An overview of the steps involved in Level 1 environmental assessment.

Table 1. Level Bioassay Current Status of Recommendation

					Ecological Assays					
	Health Effects Bioassays				Freshwater/Marine Aquatic			Terrestrial		
Characteristics	Bacteria Mutagenesis	CHO Clonal Toxicity	RAM	In Vivo Rodent Toxicity	Fish	Invertebrate	Algae	Insect Toxicity	Soil Microorganism Toxicity	Plant Toxicity
Test sample applications										
solids	+	+	+	+	+	+	+	+[a]	–	–
liquids	+	+	+	+	+	+	–	+	+	+
gases	+/–	–	–	–	–	–	–	+	–	+
mixtures (solid/liquid) and slurries	+	+	+	+/–	+/–	+/–	+/–	+/–	+/–	–
filter or sorbent extracts	+	+	+	–	–	–	–	+	–	–
Test results applicable to chronic toxicity	+	–	–	+/–	–	–	–	+/–	–	–
Bioassay includes life cycle analysis	–	–	–	–	–	–	–	+	–	–
Quality control procedures established	+	+	+	+	–	–	–	+	–	–
Test method in development state	–	–	–	–[b]	+	–	+/–	+/–	+[c]	+

[a] Depends on solubility of materials.
[b] Protocol changed from rat to mouse.
[c] No test currently proposed for Level 1.



BIOASSAY SUMMARY TABLE

Technical Directive or Project No. ______________________

Contract No. ______________________

	Health Effects Tests				Ecological Effects Tests									
					Aquatic						Terrestrial			
					Fresh Water			Marine						
Sample Identification	AMES Salmonella	RAM Cytotoxicity	CHO Cytotoxicity	Rodent Toxicity	Fish	Invertebrate	Algal	Fish	Invertebrate	Algal	Plant Stress Ethylene	Root Elongation	Insect Toxicity	Notes

ND = No Detectable Toxicity
L = Low Toxicity
M = Moderate Toxicity
H = High Toxicity

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Figure 2. Example of bioassay summary sheet.

Table 2. Definition of Effectiveness Categories

Assay	Activity Measured	Units	MAD[a]	Range of Concentration or Dosage			
				High	Moderate	Low	Not Detectable
Ames test	Mutagenesis[b]	mg/plate	5	<0.05	0.05-0.5	0.5-5	ND at >5
		µl/plate	50	<0.5	0.5-5.0	5-60	ND at >50
RAM/WI-38 and CHO toxicity	Lethality EC50[c]	mg/ml	1	<0.01	0.01-0.1	0.1-1	ND at >1
		µl/ml	600	<6.0	6.0-60	60-600	ND at >600
		µl/ml	20[c]				ND at >20
Rodent toxicity	Lethality LD50[e]	gm/kg	10	<0.1	0.1-1	1 -10	ND at >10
Aquatic Tests							
Algae	Growth inhibition EC50	gm/l	1	>0.01	0.01-0.1	0.1-1	ND at >1
		%	100	<20	20-75	75-100	ND at >100
Fish	Lethality LC50[f]	gm/l	1	<0.01	0.01-0.1	0.1-1	ND at >1
		%	100	<20	20-75	75-100	ND at >100
Invertebrate	Lethality LC50	gm/l	1	<0.01	0.01-0.1	0.1-1	ND at >1
		%	100	<20	20-75	75-100	ND at >100

[a] Maximum applicable dose (technical limitations).

[b] Negative response at 5 mg/plate or at level of toxicity is given as ND: positive response requires calculation of minimum effective concentration (MEC) to produce a positive mutagenic response; H, M, and L designations are made from MEC values of positive agents.

[c] Calculated concentration expected to produce effect in 50% of population.

[d] Volumes used for solvent exchange samples (this maximum keeps DMSO below level of toxicity).

[e] Calculated dosage expected to kill 50% of population.

[f] Calculated concentration expected to kill 50% of population.

placed directly into the bioassays: only a few filters or sorbent collectors were extracted or sonicated with solvents. A severity assessment was conducted both on chemicals detected and biological responses; a comparison of the analyses indicated generally complementary results (Sexton, 1979). In a few instances, toxicity was not predicted by the chemical analysis. Pretest processing could have a significant impact on such data. With the increased use of Level 1 testing procedures, the questions of whether to use a sample directly or to reduce it to its active constituents or to concentrate a dilute sample become more relevant.

Important components in the final data analysis and interpretation of Level 1 bioassays are

1) sampling methods (selecting a representative sample),

2) storage (maintaining proper composition and preventing degradation),

3) shipping (same as for storage), and

4) pretest handling (altering chemical composition, physical state, or preferential extraction and concentration of potential toxicants and mutagens).

Sampling methods, storage, and shipping can be grouped into a single parameter called sample history (i.e., documentation of sample handling until receipt at the testing laboratory). Pretest handling encompasses the pretest sample processing techniques conducted at the testing facilities. Table 3 describes the possible effects of each technique.

Sample History

Both the final interpretations of the test results and the ranking of emission sources according to potential hazard can be affected by sample history. Representative sampling is particularly decisive.

The environmental fate of the toxic substances in emissions, for example, profoundly influences the final assessment. An emission with extremely low volume and release rate may represent a negligible health or ecological hazard even if it is highly toxic. However, if the emission were large (e.g., of fly ash from power plants), significant human and ecosystem exposure would result (Fisher et al., 1979; Kubitschek and Venta, 1979). If a potential hazard is identified as a function of its release rate (volume per unit time), toxicity, and environmental fate, a quantified assessment may be possible. Thus, if the bioaccumulation of any

Table 3. Effects of Pretest Handling Methods

Pretest Handling	Method Employed	Possible Effect
Particulate extraction	(a) Organic solvent/sonication (b) Soxhlet/organic solvent	Preferential releases to toxic and mutagenic organic materials from bound state. If these organics are not released under normal environmental or physiological conditions, they can skew ranking scheme.
Liquid concentration	Flow through XAD-2 resin column followed by soxhlet extraction from resin and solvent exchange	Concentrates organics and permits inorganics to pass through. Some of the inorganics may be important toxicants. Again, preferential concentration of organics might increase detection of mutagens and skew ranking scheme. Concentration of chemicals may introduce artifacts by altering chemical: chemical dynamics not encountered in dilute solutions.
Extraction from collection filter	Sonication in organic solvent	Preferential release of toxic and mutagenic agents preferentially attached to filter material. Degradation and alteration of chemicals is known to occur when bound to filter. Sonication of filter may release small particles that would be toxic in RAM assay.
Solvent exchange and solubilization	Addition of sample to methylene chloride, acetone, benzene, DMSO, etc.	Preferential release of soluble compounds to the target organism. Nonrepresentative composition of original sample by extraction of organics.
Particulate sizing and grinding	(a) Filter collection (b) Grinding and sizing with mill and wire screen	Development of a physical state more amenable to cellular phagocytosis or animal absorption. This could skew ranking by producing abnormally high levels of toxicity.

single component is very large, the potential hazard could be significant. However, only toxicity is being recorded at present (see Figure 2). This factor is not being related to the type of emission release rate or to the volume of emission sample. Attempts should be made to document the history of each sample.

Pretest Handling

Several laboratories in the United States (particularly IERL of EPA in Research Triangle Park, NC, and Oak Ridge National Laboratory in Oak Ridge, TN) have initiated programs to analyze complex mixtures for mutagenic activity (Epler, 1979; Huisingh et al., 1978). The results of the Ames *Salmonella* assay serve as a preliminary indicator for further fractionation tests to determine the biologically active components (pure substances).

The Ames test procedures specify how to collect the desired sample (particulate, gas, or liquid) on a filter or solid sorbent; extract the organics from the filter or sorbent (by sonication or Soxhlet methods) into an organic solvent: exchange solvents to dimethylsulfoxide (DMSO) or evaporate to dryness and resuspend in DMSO; and conduct the bioassay on the concentrated extract. Chemical fractionation and further testing may eventually lead to specific associations between chemicals or chemical classes with biological activity (Huisingh et al., 1978). Table 3 describes some of the pretest sample processing techniques currently used and indicates their possible effect on the interpretation of Level 1 bioassay data.

The Level 1 toxicity assessments listed in the Bioassay Summary (Figure 2) include several types of biological systems and phylogenetic levels. If a significant amount of pretest processing is anticipated, all the Level 1 tests, and not just the Ames or one or two selected tests, should be evaluated. Otherwise, the data balance will be upset and the ranking of the test site might be biased by an abnormally toxic response from an extract or concentrate in a single assay, erroneously exaggerating the potential hazard. This precaution is not meant to preclude pretest processing. However, as with the sample history factor (release rate), the pretest sample processing must be factored into the final assignment of a toxicity value and its contribution to the potential hazard. A mechanism needs to be developed to normalize the data obtained from preprocessed samples. The simplest approach might be to divide the actual test response by a concentration factor.

RECOMMENDATIONS

The following recommendations would facilitate the implementation of sample history documentation and pretest sample processing and enhance the reliability of the Level 1 bioassays:

1) Documentation of sample collection, storage, shipping, and pretest processing should be available for all test samples. Examples of forms for this purpose are shown in Figures 3 and 4.

2) Pretest processing should be factored into the final toxicity designations of H, M, L, and ND. Specific methods need to be developed to normalize data obtained from samples modified prior to evaluation.

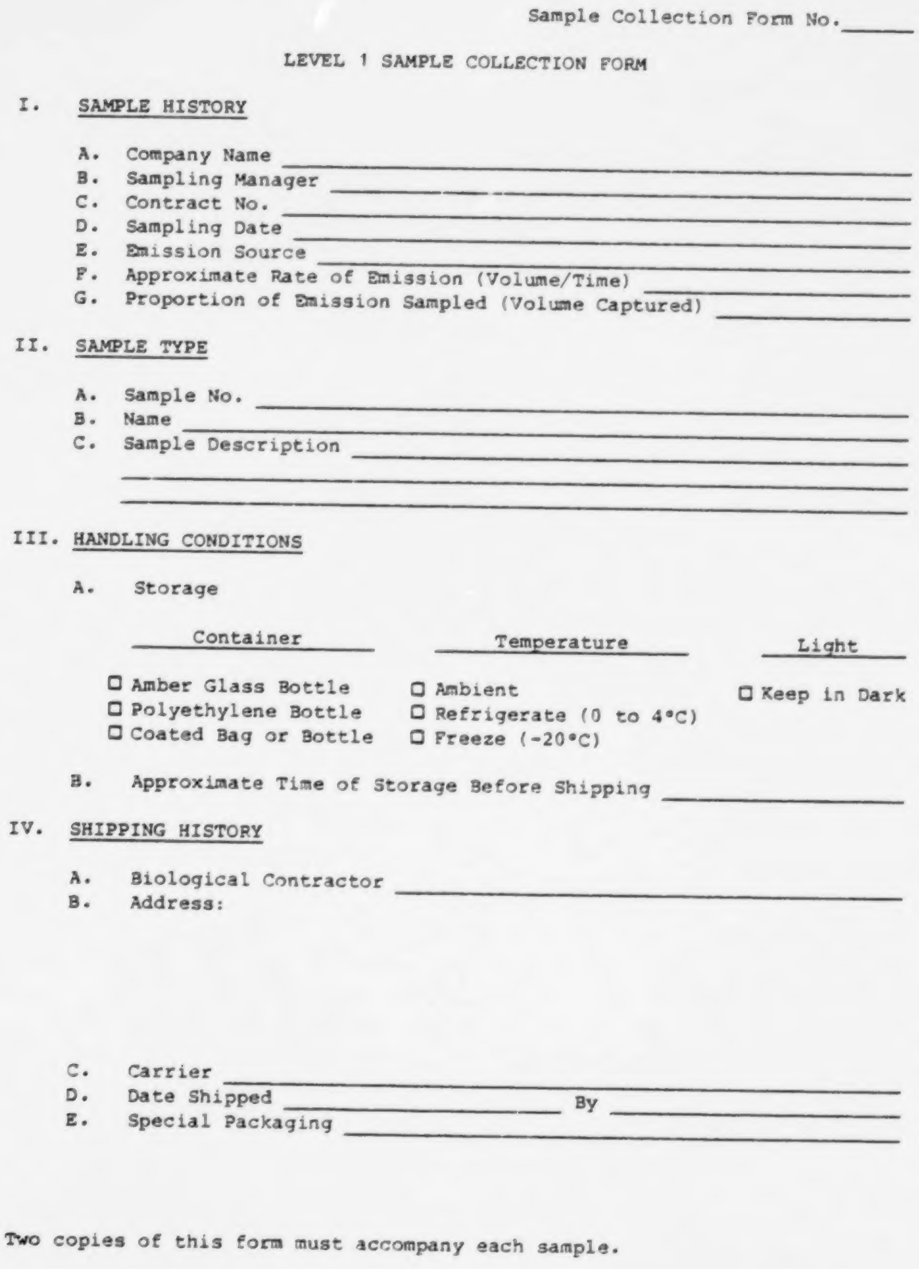
3) Emissions from a given site should be applied uniformly to the spectrum of Level 1 bioassays so as not to bias the final interpretation. Data related to environmental fate should be included.

4) Discharge-severity calculations used to factor chemical and physical information should be expanded to include biological response and fate. Specific methods need to be developed.

CONCLUSIONS

Level 1 environmental assessment bioassays should permit accurate ranking f emissions from stationary-site sources with respect to their potential hazard. Pretest processing should be kept to a minimum and applied uniformly across all Level 1 bioassays. The ranking must ensure that the potential hazard will be derived from emissions in the state in which they were released into the environment.

The Level 1 Environmental Assessment Program should include the environmental fate and emission release rate along with the chemical and bioassay toxicity determinations. This approach would result in a second level determination called a severity potential hazard. A general scheme has been proposed to develop this assessment and is given in Figure 5. If a potential hazard can be calculated with reasonable accuracy, the usefulness of Level 1 results will be greatly enhanced.



Sample Collection Form No.______

LEVEL 1 SAMPLE COLLECTION FORM

I. SAMPLE HISTORY

A. Company Name ______________________________
B. Sampling Manager ______________________________
C. Contract No. ______________________________
D. Sampling Date ______________________________
E. Emission Source ______________________________
F. Approximate Rate of Emission (Volume/Time) ____________
G. Proportion of Emission Sampled (Volume Captured) __________

II. SAMPLE TYPE

A. Sample No. ______________________________
B. Name ______________________________
C. Sample Description ______________________________
____
____

III. HANDLING CONDITIONS

A. Storage

Container	Temperature	Light
☐ Amber Glass Bottle	☐ Ambient	☐ Keep in Dark
☐ Polyethylene Bottle	☐ Refrigerate (0 to 4°C)	
☐ Coated Bag or Bottle	☐ Freeze (-20°C)	

B. Approximate Time of Storage Before Shipping ____________

IV. SHIPPING HISTORY

A. Biological Contractor ______________________________
B. Address:

C. Carrier ______________________________
D. Date Shipped ______________ By ______________
E. Special Packaging ______________________________

Two copies of this form must accompany each sample.

Figure 3. Example of Level 1 sample collection form.

Sample Processing Form No.________

LEVEL 1 SAMPLE PROCESSING FORM

I. SAMPLE IDENTIFICATION

A. Sample Collection Form No. ________________________________
B. Contract No. ____
C. Project Officer ______________________________________
D. Sample No. ____

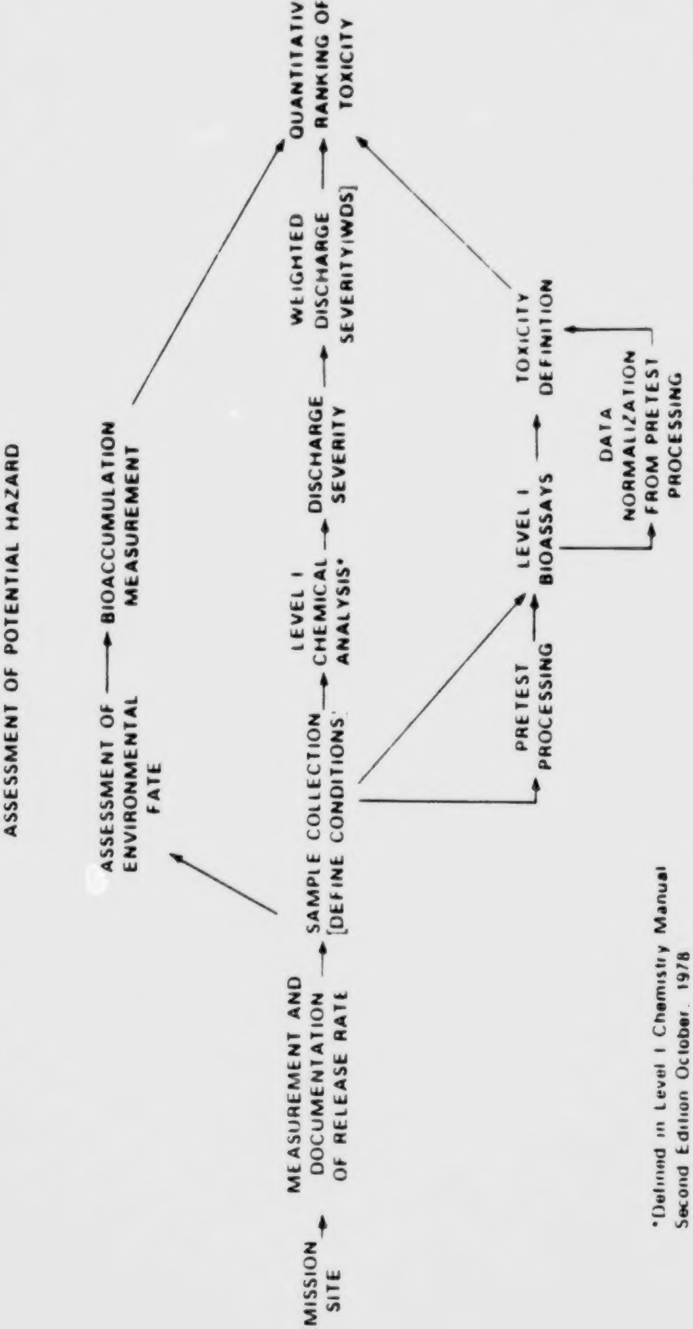
II. SAMPLE TYPE AND PROCESSING REQUIREMENTS

Basic Type	Subtype	Processing
☐ Solid	☐ Solid Granular	☐ Grind to <5μ size
	☐ Slurry (>50% Solids)	☐ Extract Particulates with Organic Solvent
	☐ Particulates from Filter	☐ Remove Particulate from Filter
	☐ Filter/Unit Particulates	☐ Prepare Water Leachate
☐ Liquids	☐ Suspensions (<50% Solids)	☐ Concentrate with XAD-2
	☐ Effluent	☐ Solvent Exchange
	☐ Leachate	☐ Evaporate to Dryness
	☐ Extract	
	☐ Condensate	
☐ Gas	☐ Pressure Collection	
	☐ Vacuum Collection	

III. BIOASSAYS REQUESTED

☐ Ames, Salmonella
☐ RAM Toxicity
☐ CHO Clonal Toxicity
☐ Rodent Quantal Toxicity
☐ Freshwater Fish Toxicity
☐ Freshwater Invertebrate
☐ Algal Test
☐ Insect Toxicity
☐ Plant Test
☐ Soil Test

Figure 4. Example of Level 1 sample processing form.


ASSESSMENT OF POTENTIAL HAZARD
EMISSION SITE
MEASUREMENT AND DOCUMENTATION OF RELEASE RATE
SAMPLE COLLECTION [DEFINE CONDITIONS]
ASSESSMENT OF ENVIRONMENTAL FATE
BIOACCUMULATION MEASUREMENT
LEVEL I CHEMICAL ANALYSIS*
DISCHARGE SEVERITY
WEIGHTED DISCHARGE SEVERITY(WDS)
QUANTITATIVE RANKING OF TOXICITY
PRETEST PROCESSING
LEVEL I BIOASSAYS
TOXICITY DEFINITION
DATA NORMALIZATION FROM PRETEST PROCESSING

*Defined in Level I Chemistry Manual Second Edition October 1978

Figure 5. Proposed scheme for a second stage evaluation of Level 1 results (defined in Lentzen et al., 1978).

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PB82-233461

BIOLOGICAL MONITORING OF FLUIDIZED BED COAL COMBUSTION OPERATIONS I. INCREASED MUTAGENICITY DURING PERIODS OF INCOMPLETE COMBUSTION

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INTRODUCTION

The Ames *Salmonella* microsome assay (Ames and Yamasaki, 1975) is used extensively to screen environmental pollutants because of its rapidity, its relatively low cost, and its sensitivity in detecting carcinogens (McCann et al., 1975). We applied the Ames assay to the study of mutagenic particulates produced by an experimental process-development fluidized bed combustor (FBC) at Argonne National Laboratory. This FBC also was used for mouse inhalation toxicology experiments (Kirchner et al., 1980; this study is reported in this volume as Biological Monitoring of Fluidized Bed Coal Combustion Operations II.).

The original plan was to determine the relative mutagenicities of the different effluents produced by the FBC and to measure the mutagenicity of the particulate effluent during the mouse exposures. However, our earliest measurements (Kubitschek and Haugen, 1980) indicated that the mutagenicity was much greater than that for a larger FBC (Clark et al., 1978). This excessive mutagenicity in the Argonne FBC was traced to operating conditions: fly ash deposited on the final filter during start-up periods was up to 60 times as mutagenic as that produced during steady operation (Kubitschek and Williams, 1980). This finding, and the variability in the mutagenicity of the fly ash produced during a 1000-h (40-day) run, made it apparent that the Ames assay might be used to examine the effects of process conditions on levels of mutagenicity.

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MATERIALS AND METHOD

The Argonne FBC, operated by the Chemical Engineering Division, is a process-development, 6-in.- (15.2-cm-) diameter combustor maintained at atmospheric pressure. This FBC burned high-sulfur (5.5%) bituminous coal (Sewickly) and used as the sorbent either of two calcitic limestones (Greer or Grove) treated with sodium carbonate. The combustor operated at a power level of 21 kW, with coal feed controlled by the concentration of oxygen in the off-gas stream. Oxygen was normally maintained at 3%, and sulfur dioxide at 700 ppm. The operating temperature was 850°C.

Particulates were collected in cyclones and also on a porous metal filter, located downstream in the off-gas line. This filter had an efficiency greater than 98% for particles 6 μm in diameter or larger. Particle cutoff diameters for the first and second cyclones were approximately 8 and 5 μm, respectively. An important design feature of the FBC was the availability of twin off-gas clean-up trains downstream from the combustion chamber, each containing a primary cyclone, secondary cyclone, and porous metal filter. The effluent was vented to the exhaust system. With this equipment, off-gas trains were used alternately to collect filter particulate samples, and equipment could be cleaned to reduce cross-contamination between the samples.

Initially, particulate samples (fly ash) were collected after runs of a single day's duration (8 to 10 h). These samples were extracted for 45 min at 37°C with dimethylsulfoxide, which yielded greater observable mutagenicity than did any of the other organic solvents tested (Kubitschek and Haugen, 1980). Metabolic activation did not increase observable mutagenicity (Kubitschek and Haugen, 1980), which agreed with similar observations by Fisher et al. (1979). In all of the experiments described below, mutagenic activity was determined with the Ames *Salmonella* assay using strain TA98 without microsomal enzyme activation.

RESULTS

Mutagenicity During Start-up and Steady Operation

In a series of tests of fly ash from single daily runs of the Argonne FBC, we observed mutagenicity levels in excess of 1000 revertants/mg (Kubitschek and Haugen, 1980; Kubitschek and Williams, 1980). These values were much greater than those obtained by Clark et al. (1978) for fly ash samples from the 18-in.- (45.7-cm-) diameter FBC at the Morgantown Technology Center. Clark et al. found that when mutagenicity was detectable, activities were less than 3 revertants/mg.

We first considered the possibility that these very different mutagenicities might be due to the different temperatures at which the samples were collected. Natusch and Tompkins (1978) predicted that the adsorption of mutagenic polycyclic aromatic hydrocarbons would increase greatly as tempertures were decreased below 150°C, and the collection temperature of the Argonne filter (70°C) was known to be somewhat less than that for the Morgantown samples (estimated to be in the range of 70° to 90°C). However, when the Argonne FBC was operated for longer periods, somewhat lower sample activities were observed, indicating that sample activities might depend on operating conditions.

To distinguish between these possible explanations, fly ash samples were collected from the first and second cyclones and the filter both during start-up periods and during steady operation. The observed mutagenicities (Kubitschek and Williams, 1980) are shown in Table 1. An inverse relationship between mutagenicity and particulate collection temperature can be seen, in agreement with the predictions of Natusch and Tompkins (1978). However, mutagenicity levels varied much more widely when operating conditions were changed, and fly ash mutagenicity during start-up of operations was as much as 60-fold greater than that observed during later steady operation. Clearly, the great bulk (> 98%) of the difference between our earlier determinations and those of Clark et al. (1978) can be assigned to excessive mutagenicity that was deposited in our samples during start-up and shut-down of operations.

Table 1. Particulate Effluent Mutagenicity During Start-up and Steady FBC Operation[a,b]

		Operation		
Site	Temperature	Start-up	Steady	Ratio
Primary cyclone	150°C	3 ± 2	0 ± 1	-
Secondary cyclone	95°C	470 ± 25	8 ± 1	59
Filter	70°C	1400 ± 20	22 ± 2	64

[a] From Kubitschek and Williams, 1980, by permission of the publisher.

[b] Values are averages ± standard errors for net numbers of TA98 His$^+$ revertants/mg ash extracted.

While the data shown in Table 1 are those for the experiment with the greatest difference between start-up and steady operating conditions, the same qualitative results were obtained in each of three experiments. These results suggest that excessive mutagenicity might be produced during periods of incomplete combustion, as would be expected during start-up of combustion (Kubitschek and Williams, 1980).

Mutagenicity During Departures from Steady Operation

Particulate mutagenicity was monitored for samples collected daily during a 1000-h exposure of mice to gaseous and particulate effluents from the FBC. During this period, the specific mutagenicity of the fly ash varied widely (Figure 1) with unusually high levels of mutagenic activity produced during six transient periods. Later, by examining the record of operations, we found that each of these peaks of mutagenic activity (Table 2) was associated with a departure from steady combustor operation, due either to start-up of operations or to mechanical breakdown (Kubitschek et al., 1980).

Examination of the gas concentration records indicated that carbon monoxide (CO) concentrations also increased during the same periods of high fly ash mutagenicity. Figure 1 shows the sum of the peak values for CO concentrations in excess of 1000 ppm during each 12-h period of the run. This level was chosen because mutagenicity did not appear to be closely correlated with the CO peaks of smaller magnitude, which became more frequent as CO concentrations approached the background level of approximately 500 ppm. Good correlation between this peak CO concentration and sample mutagenicity is evident in Figure 1; the correlation coefficient is 0.72.

Correlations in time and in intensity for the individual transients supported the correlation between mutagenicity and peak CO concentrations. The mean time of occurrence of mutagenicity in each peak and the mean time of peak CO production for the corresponding periods are shown in Figure 2 and Table 2. The coefficient of correlation between the mean times of occurrence was 0.9998. This close correlation is especially noteworthy considering that the average duration of these six transient periods was more than three days, while the average deviation between corresponding CO and mutagenicity peaks was less than 5 h.

Good correlation also was observed between the magnitude of CO peak production and mutagenicity (shown in Figure 3 and Table 2); the correlation coefficient was 0.81. The average net number of revertants per milligram produced per CO value at the standard level of 1000 ppm CO was 16.4 (standard error = 2.1). Thus, both

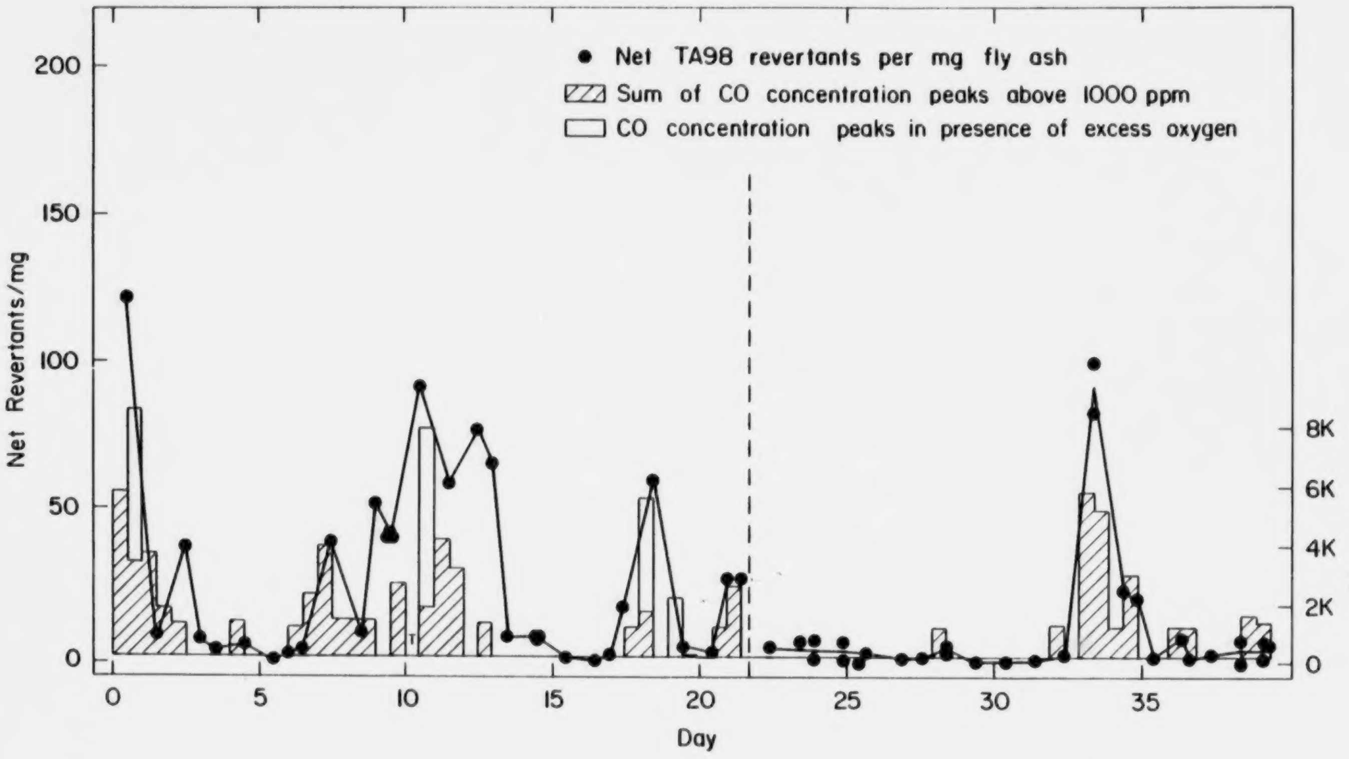

Figure 1. Fly ash mutagenicity and peak CO concentrations during a 40-day period of FBC operation (Kubitschek et al., 1980). The bar graph shows the sum of the CO concentration peaks for each half-day period. The letter T (day 10) indicates a large decrease in operating temperature. The dashed line indicates a period of about 10 days during which the FBC was shut down for repairs.

Table 2. Mutagenicity and CO Production During Departures from Steady FBC Operation[a]

Period (days)	Mean Time (days)[b] CO Peaks	Mean Time (days)[b] Mutagenicity	CO (ppm x 10^{-3})	Mutagenicity (rev/mg)	Ratio[c]
0-2	0.55	0.58	21.2	274	12.9
6-8	7.42	7.67	11.7	126	10.8
9-13[d]	11.60	11.35	18.4	458	24.9
17-19	18.50	18.09	8.4	154	18.3
20-21	21.10	20.97	3.7	56	15.1
32-35	33.80	33.63	14.4	237	16.5
				Mean:	16.4 ± 2.0

[a] From Kubitschek et al., 1980.

[b] The mean time of occurrence for each transient increase in CO production (CO peaks) and in mutagenicity is given in days numbered sequentially during the tested periods.

[c] Revertants per milligram: ppm CO x 10^{-3}.

[d] Values for the low temperature period on day 10 are excluded.

the time of occurrence and the degree of mutagenicity of these transient periods of departure from steady operation were correlated with increased concentration of CO in the combustion chamber.

CONCLUSIONS

The observed correlations strongly support our earlier suggestion (Kubitschek and Williams, 1980) that excessive effluent mutagenicity occurs during incomplete coal combustion. The increase in mutagenicity during periods of incomplete combustion might have been a result of the evolution of adsorbed hydrocarbons (especially during start-up) or, alternatively, the result of only partial oxidation of the macromolecules of which coal is composed, with the release of complex hydrocarbon moieties that are mutagens or are capable of forming mutagens. Preliminary results for chemical characterization of mutagens in fly ash samples from the Argonne FBC are consistent with either hypothesis: several classes of mutagenic compounds were identified, including 2- to 6-ring aromatics, phenols, carbonyls, alcohols, and carboxylic acids, and other very polar compounds (Kubitschek and Haugen, 1980), and the

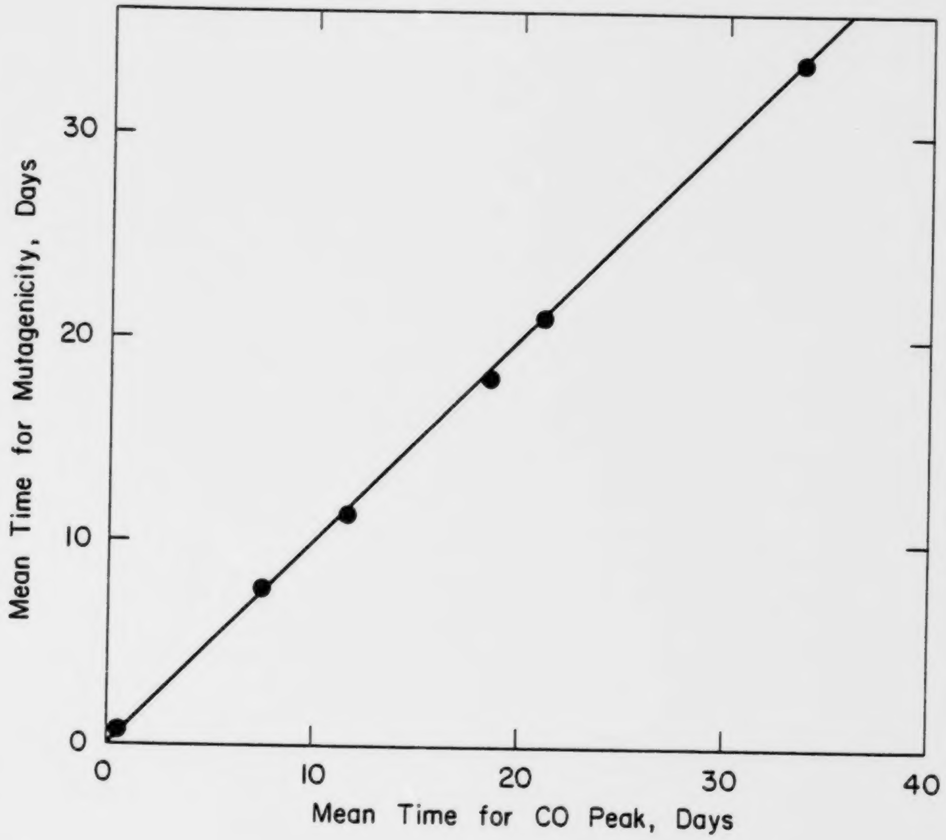

Figure 2. Correlation between mean times of occurrence of increased CO production and mutagenicity during transient departures from steady FBC operation (redrawn from figure in Kubitschek et al., 1980).

amounts of these mutagens decreased during steady operation of the combustor (Kubitschek and Williams, 1980).

These results could influence estimates and comparisons of the biological risk from the various coal technologies. If mutagenicity increases with incomplete combustion, then any estimate of biological hazards or comparison among different coal technologies should take into account the mutagenic activities associated with both steady and nonsteady operating conditions. If, as in our experiments, mutagen production occurs primarily during start-up periods or other transient departures from steady

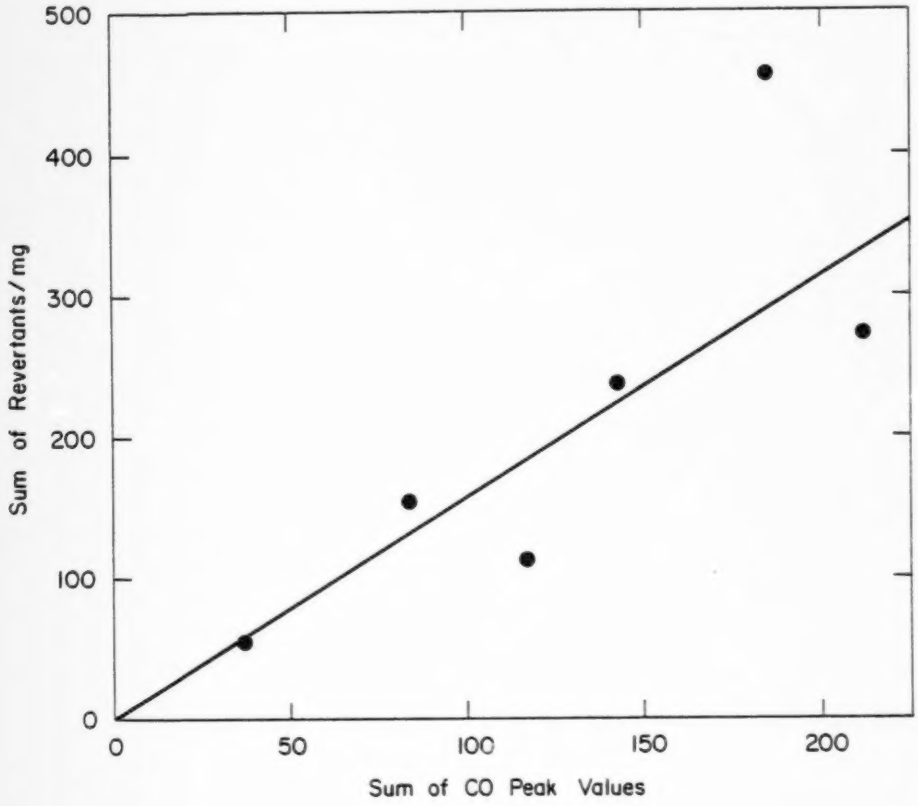

Figure 3. Correlation between specific mutagenicity and the sum of the CO concentration peak values (redrawn from figure in Kubitschek et al., 1980).

operation, then biological risk would be determined mainly by the frequency and duration of those operational transients.

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BIOLOGICAL MONITORING OF FLUIDIZED BED COAL COMBUSTION OPERATIONS II. MAMMALIAN RESPONSES FOLLOWING EXPOSURE TO GASEOUS EFFLUENTS

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INTRODUCTION

Several coal combustion technologies, including fluidized bed combustion, have potential for meeting government-imposed environmental pollution guidelines. Fluidized bed combustion enables the combustion of high sulfur coal while limiting the emission of sulfur dioxide.

In a previous study (Kirchner et al., 1980), detrimental biological effects were observed in animals exposed to whole (gaseous and particulate) effluents derived from the atmospheric pressure fluidized bed coal combustor (FBC) operated by the Chemical Engineering Division at Argonne National Laboratory (ANL), Argonne, IL. In the study presented here, the animals were exposed only to gaseous FBC components in order to determine which effluent components are responsible for these observed biological effects. Three testing systems were applied.

To assess pulmonary alveolar macrophage (PAM) function, one of the organism's first lines of defense against airborne pollutants, Brennan et al. (1980) modified the assay of neutrophil function first described by Tan et al. (1971). The assay differentiates between defective phagocytosis and impaired intracellular killing. Effective PAM function is required to eliminate inhaled micro-organisms, particulate pollutants, and other inhaled particles.

Increased numbers of PAMs were observed in the lungs of animals exposed only to FBC fly ash (Brennan et al., 1980), taxing the lung macrophage progenitor stem cell compartment. To assess the systemic toxic effects following acute exposure to FBC gaseous effluent, the Till and McCulloch spleen colony assay (1961) was used. The lungs and other tissues thought to be at risk were also examined histopathologically during and after the exposures.

METHODOLOGY

The process-development-scale atmospheric pressure FBC described by Kirchner et al. (1980) was used to expose mice and rats to gaseous effluents of fluidized bed combustion. The effluent was diluted in two stages by an effluent dilution system. The effluents were taken from a point downstream of the final filter particle cleanup system, to eliminate the particulate component of the effluents (Figure 1). Following a 20-fold dilution, the effluents were delivered to the top of an Atmospheric Effects Simulator (AES)(Figure 2) that exposed the diluted effluent to simulated sunlight for 15 min. The animals were thus exposed to diluted gaseous combustion effluents only. The levels of carbon monoxide, nitrogen monoxide, sulfur dioxide, nitrogen oxides, and total vapor-phase hydrocarbons were monitored instrumentally in the exposure chambers throughout the experiments.

PURPOSE

Two experiments, each with a 500-h (~ 21-day) continuous exposure period, were conducted. In Experiment I, 154 male $B6CF_1$/Anl mice, each 120 days old, and 8 male Fisher F-344 rats, each 90 days old, were exposed in chambers designed and built in this laboratory (Kirchner et al., 1980; Figure 3). At the same time, similar control animals (66 mice and 8 rats) were placed in identical chambers through which only HEPA-filtered room air was passed. In Experiment II, previously exposed animals (88 mice and 8 rats) were re-exposed for a second 500-h period. In addition, previously untreated animals (88 mice and 8 rats) were exposed for 500 h. The two control groups in Experiment II were also exposed to HEPA-filtered room air: one group consisted of 44 control mice from Experiment I, and the other 44 untreated mice. The animals were maintained on a cycle of 12 h light and 12 h darkness. They had food (Wayne Lab Blox, Allied Mills) and water *ad libitum*. The animals' condition was checked daily.

The animals were exposed to gaseous effluents obtained only during steady-state operation conditions. Steady state is defined by the following conditions: 1) a period at least 12 h after initiation of coal feed; 2) sulfur dioxide concentrations in the undiluted off-gas at a constant 700 ± 50 ppm; 3) a bed temperature of 850 ± 5°C; and 4) carbon monoxide, nitrogen monoxide, and total vapor-phase hydrocarbon concentrations stabilized.

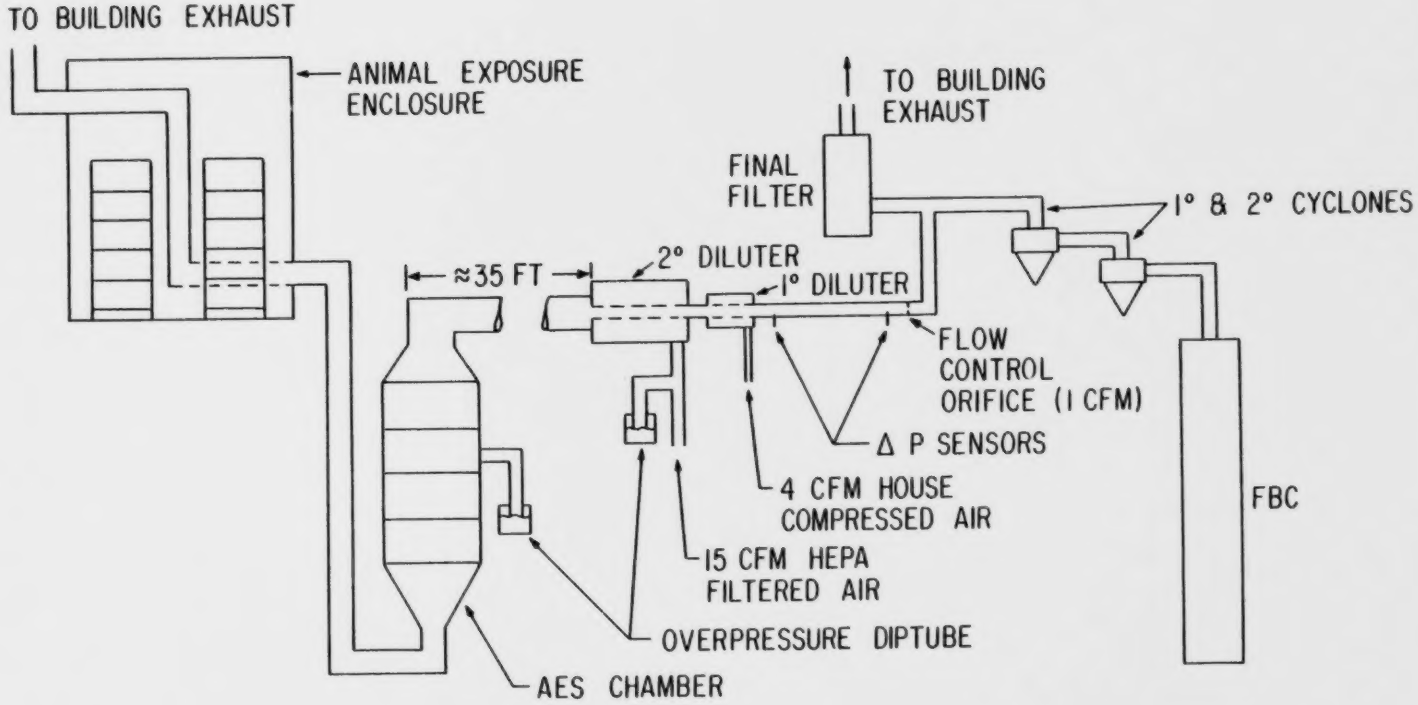
TO BUILDING EXHAUST
ANIMAL EXPOSURE ENCLOSURE
TO BUILDING EXHAUST
FINAL FILTER
1° & 2° CYCLONES
≈35 FT
2° DILUTER
1° DILUTER
FLOW CONTROL ORIFICE (1 CFM)
Δ P SENSORS
4 CFM HOUSE COMPRESSED AIR
15 CFM HEPA FILTERED AIR
OVERPRESSURE DIPTUBE
AES CHAMBER
FBC

Figure 1. Schematic diagram of the FBC and the associated effluent delivery system, the AES (reproduced from Kirchner et al., 1980).

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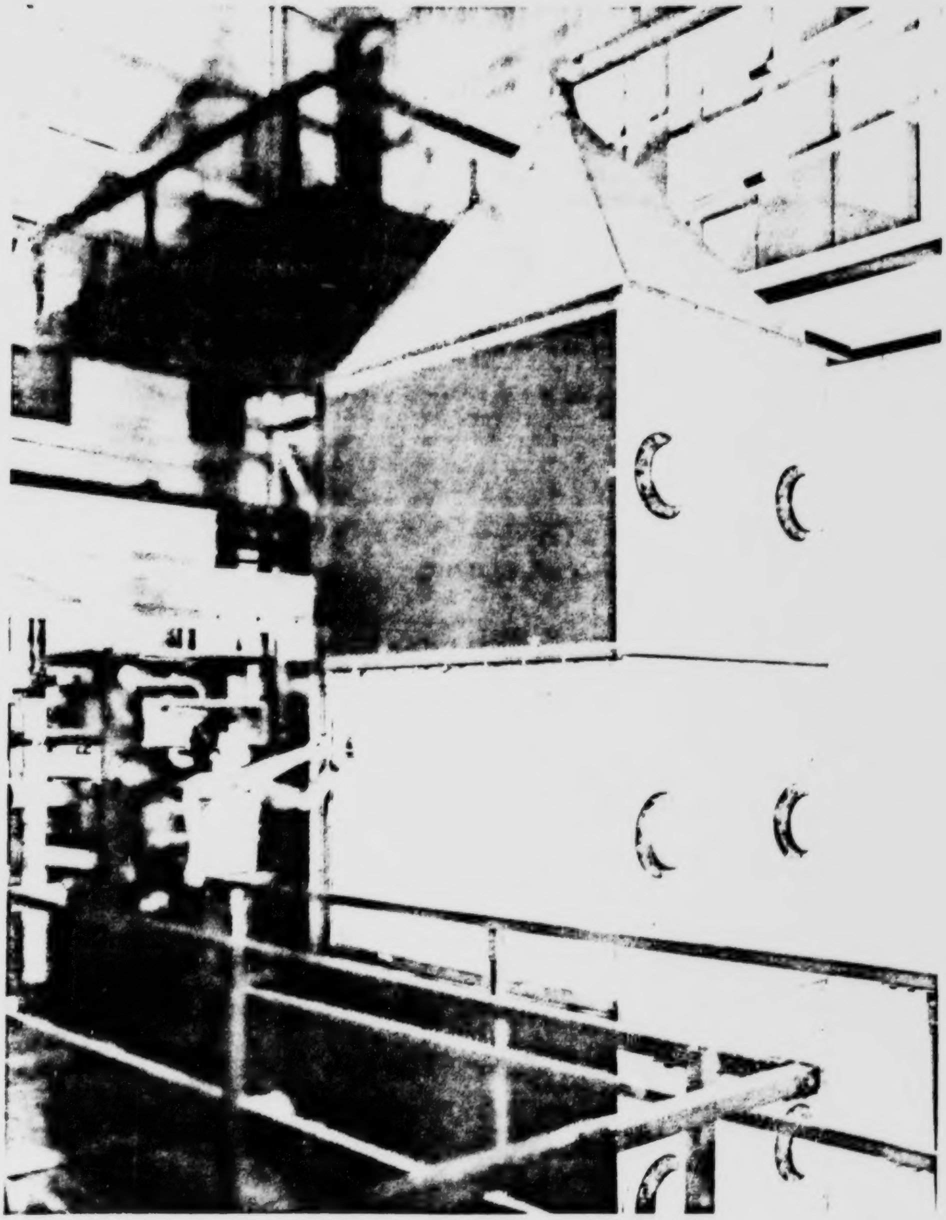

Figure 2. AES with one of the protective covers holding the aluminum reflectors removed. Note sample ports on far side of the chamber used for collecting gaseous and particulate samples from the AES (reproduced from Kirchner et al., 1980).

Figure 3. Animal exposure system shown with doors to aluminum enclosure removed (reproduced from Kirchner et al., 1980).

To verify that the animals were exposed only to the gaseous component of the effluent, the concentration of particulate in the diluted off-gas was monitored with optical and electrical aerosol analyzers. For the smallest-diameter particles (0.01 to 1.0 μm) the Thermo Systems, Inc., electrical aerosol analyzer was used. For particles in the range of 0.3 to 3.0 μm or greater, the Royco forward-light-scattering particle counter was used. These analyses also indicated the efficiency of the particle cleanup system.

After 250, 500, and 1000 h of exposure, two randomly selected animals from each group were sacrificed by cervical dislocation. Tissues from the lung, liver, kidney, spleen, and heart were fixed, stained with hematoxylin and eosin, and examined histologically. Four to eight days after termination of exposures, the PAM assays (from mice and rats), femoral bone marrow spleen colony assays (mice only), and histopathological examinations (mice only) were performed.

RESULTS

The levels of particulate effluent measured by the electronic particle counters were less than 10% of those observed during exposures in which the effluent was taken from a point just before the final filter of the particle cleanup system. No particles larger than 1.0 μm were detected.

The combustion-gas concentrations during the two experiments, given in Table 1, are similar to those previously reported by Kirchner et al. (1980). The only toxicant above the human threshold limit value (TLV)(ACGIH, 1977) was sulfur dioxide (TLV = 5 ppm)(animals exposed to 22 ± 5.1 ppm). The high value for sulfur dioxide resulted from the 20-fold dilution factor; the concentration would have been below the TLV following atmospheric dilution. (Note: TLVs are based on time-weighted average concentrations to which workers can be exposed for a normal 8-h workday or 40-h workweek. The mice, however, were exposed continuously during the 500-h exposures.)

In Experiment I, three experimental mice died (1.5% of the number entering the experiment); the deaths occurred at days 6, 11, and 13 of exposure. In Experiment II, four effluent-exposed mice died, two from the 500-h exposed group (one at day 12 and one at day 16) and two from the 1000-h exposed group (both on day 16). These mice represented 2.3% of the animals entering the experiment from each group. No moribund animals were observed and no rats or control animals died during either experiment.

Table 1. Concentration of Gaseous Effluents in the Atmospheric Effects Simulator During a 500-Hour Exposure[a]

Gas	Concentration (ppm)[b]	TLV (ppm)[c]
Carbon monoxide	17 ± 11	50
Nitrogen monoxide	19.0 ± 6.3	25
Nitrogen oxides	1.1 ± 0.1	5
Sulfur dioxide	22.0 ± 5.1	5
Total hydrocarbons (vapor phase)	2.1 ± 0.75	--

[a]Averages of readings taken every 6 h during the 500-h exposure ± 1 standard error).

[b]Determined by instrumental gas analyzers.

[c]Threshold limit values for chemical substances in workroom air adopted by the American Conference of Governmental Industrial Hygienists (1977).

Four to eight days after the termination of the experiments, PAMs were taken from both rats and mice. No impairment was found in their ability to engulf and kill a challenge dose of Staphylococcus aureus (Table 2).

The spleen colony assay showed that a 500-h exposure to the effluent increased spleen colony formation 167% over control values. The sizes of the spleen colonies from mice exposed 500 h were generally larger than those from the control mice. In mice exposed for 1000 h, however, the number of spleen-colony-forming units (CFUs) was comparable to the control value (Table 3).

Histological changes in the lungs of exposed mice were limited to a slight accumulation of fly ash in the macrophages in mice sacrificed after 500 and 1000 h of exposure. Fly ash was not present in the control mice or the animals exposed for 250 h. These histological changes were mild compared with all levels and times of exposure for previous exposures to the whole effluent (Kirchner et al., 1980). No evidence of epithelial hyperplasia or obvious proliferation of macrophages was seen. The gross and histological appearances of all other organs were normal.

Three days after termination of Experiment II, the 500- and 1000-h exposed mice had lost 10% of their initial body weights, while the control mice had insignificant weight loss (< 1%). The rats, too, had moderate weight loss (500-h exposed, 9%; 1000-h

Table 2. Pulmonary Alveolar Macrophage Function Four to Eight Days After *In Vivo* Exposures to Effluents[a]

Experiment		Treatment	Duration of Exposure (h)	*Staphylococcus aureus* Phagocytized & Killed (%)	*Staphylococcus aureus* Phagocytized but Not Killed (%)
I.	Mice	control	500	98.7	0.2
		exposed	500	99.3	0.0
	Rats	control	500	95.1	1.5
		exposed	500	93.8	1.8
II.	Mice	control	500	99.9	0.0
		exposed	500	98.2	0.1
		exposed	1000	99.9	0.0
	Rats	control	500	93.8	1.5
		exposed	500	97.6	0.3
		exposed	1000	98.1	0.7

[a] Pooled alveolar macrophages from 12 mice/group; results are expressed as the percent of the total challenge dose of bacteria.

Table 3. Hemopoietic Colony-forming Units (CFUs) in Mice Four to Eight Days After Termination of Exposures to FBC Effluents[a]

Treatment	Duration of Exposure (h)	CFUs/10^5[b] Nucleated Cells
Control	--	46.5 ± 3.65
Exposed	500	78.0 ± 4.16
	1000	41.6 ± 2.50

[a] For each determination, pooled femoral bone marrow cells (2.0×10^4/recipient) from two donors were injected into 15 irradiated recipient mice.

[b] Mean number of spleen colonies per mouse from 15 mice (± 1 standard error).

exposed, 13%), with a negligible (< 2%) weight loss in the controls.

CONCLUSIONS

In the previous study (Kirchner et al., 1980), exposure of the rodents to whole (gaseous and particulate) FBC effluent for 500 h significantly impaired the PAMs' ability to phagocytize and kill a challenge dose of Staphylococcus aureus. However, after an exposure of 1000 h, the exposed animals' macrophages appeared to accommodate the exposure conditions and were again able to engulf and kill the bacteria at the same level as those of the control animals. In the present studies with FBC effluent gas only, the bacterial cell killing was unimpaired in either the 500- or 1000-h exposure groups, indicating that the temporary reduction in macrophage function was probably due solely to the high level of particulate matter.

The spleen colony assay results indicated that exposure to the gaseous effluent for 500 h acted as a strong proliferation and differentiation stimulus to the pluripotential stem cells. The numerous colonies represent large numbers of proliferating pluripotent stem cells; the large size of the nodules indicates the rapidity of the proliferative response of these cells to the stimulus (McCulloch, 1970). Prolonged exposures (1000 h) resulted in CFUs similar to those of unexposed mice in both colony size and numbers of colonies. These results differed from those observed in the previous work with particulate effluents (Kirchner et al., 1980), in which there appeared to be a cumulative toxic effect (i.e., decrease in CFUs) in the longer exposures, with little, if any, recovery or adaptation to the effluent exposures. The observed decrease in CFUs from 500 to 1000 h of exposure may have been due to a depletion of the stem cell compartment resulting from the strong persistent stimulus (500-h exposure) for stem cell proliferation (Till, 1976). The initial demand for rapid proliferation may have reduced the pluripotent stem cell pool, perhaps simultaneously decreasing the committed stem cell pool. The systemic stress on the hematopoietic stem cell compartment may ultimately impair one or more of the functional end cells. This possible alteration of hemopoiesis is currently under investigation.

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IN VITRO AND *IN VIVO* EVALUATION OF POTENTIAL TOXICITY OF INDUSTRIAL PARTICLES

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INTRODUCTION

Alveolar macrophages (AM) protect the lungs by phagocytosis and digestion of inhaled irritant particles and infectious agents. Reduced activity of the AM system can impair the lung's defensive capacity and increase susceptibility to respiratory disease. Since resistance to infection may be lowered by exposure to an inhalation hazard, changes in the major functional characteristics of AM can be used to monitor environmental stresses in the intact animal. In addition, since these cells can be obtained easily by tracheobronchial lavage and maintained in culture, they are frequently used in in vitro cellular toxicology to assess the potential inhalation hazard of various substances.

The advantages of in vitro screening assays in terms of cost and time efficiency are well known. The rabbit alveolar macrophage (RAM) test, a rapid, efficient in vitro assay, has been used extensively by the U.S. Environmental Protection Agency (EPA) and in our laboratories to compare the cytotoxicity of a variety of soluble compounds and particulate materials (Aranyi et al., 1977, 1979; Mahar, 1976; Waters et al., 1974a, b: 1975a, b: 1978). This system is capable of rapid screening and toxicity ranking of test materials and thereby identifies not only the potentially hazardous but also the inert agents. Based on the in vitro results, the number of samples to be studied further in vivo can be reduced considerably.

The purpose of these studies was to determine whether in vitro exposure of AM to various complex industrial particles produced the same relative toxicity ranking as inhalation exposure to aerosols of these particles in vivo. Our main objective was to establish the correlation between the effects of in vitro and in vivo exposures and to determine whether inhalation hazards could be predicted on the basis of in vitro screening assays.

MATERIALS AND METHODS

Particulate Stationary Source Samples

The particles were collected as baghouse samples, from electrostatic precipitators (ESP) or by cyclone sampling train from various stationary point sources, including four coal-fired power plants (three conventional and one fluidized-bed process), a steel foundry, and an aluminum and a copper smelter. Because of the large sample requirement for the aerosol inhalation exposure studies, it was not possible to provide sufficient amounts of samples collected by special emission source samplers located beyond the normal in-plant control devices. Thus, the samples reported here were not true emission or effluent samples and were not necessarily similar in composition or toxicity to emission samples.

Foundry, smelter, and selected coal combustion samples (fly ash nos. 1, 2, and 3) were provided and inorganic analysis of these particulate materials was performed by EPA Industrial Environmental Research Laboratory (IERL) at Research Triangle Park, N.C. The conventional power plant coal fly ashes (nos. 1, 3, and 4) originated from high-sulfur-containing Eastern coals, and nos. 1 and 3 were collected as baghouse samples. Fly ash no. 4, a < 3.3-μm aerodynamic size fraction of an ESP hopper fly ash, has been studied in depth by Griest and Guerin (1979) and contains arsenic, barium, cobalt, chromium, copper, lead, strontium, and zinc as some of the more prevalent trace metals, as well as traces of polycyclic aromatic hydrocarbons. The no. 2 fly ash was collected at 840°C (1550°F) from the second cyclone of a calcium-oxide-fluidized-bed coal combustion process in an experimental demonstration plant. Because of the high collection temperature, residual organic compounds were < 0.1%. Major inorganic components identified by spark source mass spectroscopy were calcium, magnesium, iron, silicon, and sulfur. The steel foundry particles were collected as a baghouse sample. No extractable organics were found, and the major inorganic constituents were iron, silicon, magnesium, and zinc. The copper smelter dust was collected by ESP at 200°C (400°F). No organic components have been identified, but trace metals such as lead, arsenic, copper, iron, antimony, and zinc were found in high concentrations. The aluminum smelter sample, collected as a baghouse dust, contained 3 mg/g of extractable organic compounds, which were mostly fused aromatics. No information on the inorganic constituents was available.

All collected samples were air-classified, and only the particles in the size-fraction of < 3 μm were used in the experiments. Since our previous studies (Aranyi et al., 1979) demonstrated that the smallest particles had the most deleterious effects on AM *in vitro*, we wanted to explore the *in vivo* correlation for this size range in particular.

In Vitro Methods

The *in vitro* experimental procedures have been described previously in more detail (Aranyi et al., 1979). Briefly, AM obtained from rabbits by tracheobronchial lavage were centrifuged and washed in Hanks' Balanced Salt Solution (HBSS), and total and differential cell counts and viability were determined. AM suspensions and separate particle suspensions at twice the projected exposure concentrations were prepared in Medium 199/HBSS supplemented with serum and antibiotics, and equal volumes of the two suspensions were mixed. The final concentration of AM in the test suspensions was maintained constant at 10^6 AM/ml: the concentration of the particles was increased stepwise to 1000 μg/ml to attain a dose-related change in viability from approximately 20 to 90%. The test suspensions were incubated in wells of disposable plastic cluster dishes placed on a rocker platform for 20 h at 37°C in a humidified 4% CO_2 atmosphere. Immediately after incubation, percent viability was determined by dye exclusion. The test suspensions were subsequently washed, centrifuged, and resuspended in HBSS before total cellular protein and adenosine triphosphate (ATP) levels were monitored. An aliquot was treated with sodium deoxycholate, the resulting cell lysate was centrifuged at 10,000 x g, and the supernatant was used for the Lowry protein assay. A second aliquot was used for ATP determination; after extraction from the cells with dimethylsulfoxide (DMSO), ATP was determined through the luciferin-luciferase reaction in a Dupont 760 Luminescence Biometer.

In Vivo Methods

Inhalation exposures. All inhalation exposure facilities are located in rooms maintained under negative pressure relative to outside areas. The animal exposure chambers as well as the aerosol generation and dilution systems are housed in second chamber enclosures (safety cabinet-glove box) that permit safe handling of the animals and maintenance and monitoring of the experimental environment.

Animals were exposed to the experimental environment in Plexiglas chambers of various sizes (87 to 476 liters) that can hold up to 240 mice in individual compartments of wire cages. The compressed air supplied to the exposure chambers for dilution or dissemination of the test agents was passed through appropriate filter systems to dry the air and remove all traces of oil and particulates. Ambient temperature and humidity were maintained throughout the exposures by providing an adequate flow of conditioned air.

Aerosols of the coal fly ash and the copper smelter dust were generated with a Wright Dust Feeder. Mass concentration was monitored optically, using a Phoenix JM7000 Aerosol Smoke and Dust Photometer, and gravimetrically, by weighing particles collected on membrane filters on an analytical microbalance and measuring the air volumes sampled by a gas meter for the corresponding time intervals. Aerosol particle size distribution was monitored by an ASAS-300A Active Scattering Aerosol Spectrometer (Particle Measuring Systems, Inc.).

The infectious aerosol was generated with a Model 841 DeVilbiss nebulizer using Streptococcus pyogenes (Lancefield Group C), grown in Todd Hewitt Broth from stock cultures obtained from colonies isolated from the hearts of infected mice. For the bactericidal activity assay, aerosols of ^{35}S-labeled K. pneumoniae were disseminated with a Retec X-70 disposable nebulizer. Aerosol particle size produced by both nebulizers was between 1 and 5 µm MMD.

Radiolabeled K. pneumoniae were cultured in modified Anderson's medium in which the sulfate requirement of the bacteria was provided by ^{35}S-labeled sodium sulfate. Before aerosolization, the bacteria were repeatedly washed and centrifuged for removal of unattached radiolabel. Bacterial counts were determined in a Petroff-Hauser counting chamber by dark field microscopy and also by culture plate technique. Radioactive counts were measured with a Mark III Liquid Scintillation System Model 6880 (Searle Analytic Inc.).

Health-effect assays. Groups of 4- to 6-week-old female CD_1 mice (Charles River Laboratories) were exposed to either aerosols of the test particles at 2 mg/m^3 mass concentration or to filtered air for 3 h/day, 5 days/week, for one, two, or four weeks. Health-effect assays followed within one hour of the last exposure.

Pulmonary free cells were obtained from mice by tracheobronchial lavage. Total cell counts were made in a hemocytometer, and differential counts were made of smears of cells fixed in methanol and stained with Wright's stain. Viability was determined by dye exclusion. Cellular ATP concentration in the lavaged cells was monitored, using a Dupont 760 Luminescence Biometer.

Pulmonary bacterial activity was determined in the lungs of individual animals through a modification of the method of Green and Goldstein (1966), whereby mice exposed to particles and control mice exposed to filtered air are challenged with radiolabeled live bacteria. The ratio of the viable bacterial count to the radioactive count in each animal's lung gives the rate at which bacteria are destroyed by the lung in a given time after infection. The streptococcus infectivity model (Ehrlich, 1966; Gardner, 1979) was used to determine the effect of particle exposure on susceptibility to respiratory infection. Groups of exposed and control mice were simultaneously challenged with streptococcus aerosol. After the challenge, the mice were removed to a clean-air isolation room, and mortality rate and survival time were recorded over a 14-day holding period.

RESULTS

In Vitro Tests

The effects of *in vitro* incubation of the various particulate samples on rabbit AM were monitored in dose-response experiments, in which cell viability (percent), total protein (micrograms, percent of control), and ATP (femtograms ATP per microgram protein, percent of control) were measured. The means and standard errors for these parameters were calculated from six or nine replicates at each concentration, with triplicate assay determinations of each replicate. When regression analysis was applied to these data, highly significant negative linear dose-response relationships were observed for each parameter in all samples (P was generally < 0.001 and occasionally < 0.01).

The estimated concentrations that were required to reduce the experimental parameters to 50% of the control responses were calculated from the linear regressions. From these data, the samples could be ranked by relative toxicity, as shown in Table 1. Two of the samples examined, those collected from the copper and aluminum smelters, were highly cytotoxic; the copper smelter sample was the more toxic of the two. Three of the power plant coal fly ashes (nos. 4, 3, and 2) showed intermediate-to-low cytotoxicity, and the last two samples had very little effect on AM. In fact, the EC_{50} values for fly ash no. 1 and the steel foundry particles could be obtained only by extrapolation above the tested concentration range for all three experimental parameters; in the cases of fly ashes nos. 2 and 3, this was necessary only for viability.

Table 1. Concentration of Particles Required to Reduce Alveolar Macrophage Viability, Total Protein Content, and ATP Levels to 50% (EC_{50})[a]

	EC_{50} (μg/ml)		
Sample	Viability	Total Protein	ATP/Protein
Steel foundry particles	>1000[b]	>1000[b]	>1000[b]
Coal fly ash no. 1 (conventional)	>1000[b]	>1000[b]	>1000[b]
Coal fly ash no. 2 (fluidized-bed)	>1000[b]	952	537
Coal fly ash no. 3 (conventional)	>1000[b]	930	553
Coal fly ash no. 4 (conventional)	949	856	445
Aluminum smelter dust	114	139	60
Copper smelter dust	11	6	5

[a] Estimated by linear regression analysis for experimental parameters expressed as: viability, %: total protein (μg), % of control; and ATP/protein (fg/μg), % of control.

[b] Highest concentration tested.

The samples collected from the copper and the aluminum smelters were not only much more toxic than the other samples, but also behaved differently. At exposure concentrations between 250 and 1000 μg/ml (used for all other test particles), they produced initial large decreases in the experimental parameters that remained fairly constant over this entire range. Only at concentrations below 250 μg/ml could a monotonic decrease in the parameters be observed with increasing exposure concentration.

The high cytotoxicity of the copper and aluminum smelter samples at low concentrations and the absence of a dose-dependent response at higher concentrations, suggested that soluble compounds released continuously into the medium during the incubation period produced these results, in addition to the particles per se. To substantiate this hypothesis, the test particles were preincubated without AM in the maintenance medium at the highest exposure concentration (1000 μg/ml), under similar conditions (20 h at 37°C) to those used in the cytotoxicity experiments. After incubation, the particles were removed from the media by ultracentrifugation and Millipore filtration (0.22-μm pore size). These filtered culture media were subsequently incubated for 20 h at 37°C with AM, and the viability of the cells was compared with that of the unexposed control AM. The viability of the cells exposed to the supernatant fractions from the copper and aluminum smelter dust samples was substantially lower than that of the unexposed control cells, demonstrating that soluble cytotoxic components were released from these samples during incubation (see Table 2). No such difference in viability was found for coal fly ash and steel foundry particles, indicating that if any compounds were solubilized from the particles of these samples, the amounts were not toxic to the AM.

Table 2. Examination of Test Particles for Soluble Cytotoxic Components by Alveolar Macrophage Viability[a]

Sample	Viability (%)
Control	96.0 to 98.0
Steel foundry particles	97.5
Coal fly ash no. 1 (conventional)	98.3
Coal fly ash no. 2 (fluidized-bed)	97.3
Coal fly ash no. 3 (conventional)	96.7
Coal fly ash no. 4 (conventional)	97.0
Aluminum smelter dust	46.8
Copper smelter dust	65.1

[a] Determined after exposure at 37°C for 20 h to the particle-free medium separated from the preincubated particles. For details, see text.

Spark-source mass spectroscopic analysis by EPA of the copper smelter particles showed that major trace metal constituents with concentrations ranging up to 20% by weight were lead, arsenic, copper, iron, antimony, and zinc, with the arsenic as high as 13%, present mostly in water-soluble form. Thus, soluble arsenic could be one of the components responsible for the cytotoxicity of the copper smelter particles. No information is now available on the solubility of the other trace metals in this sample, nor on similar properties of the aluminum smelter dust.

Thus, the *in vitro* RAM test has enabled us to evaluate the relative cytotoxicity of seven industrial particulate samples. The data have demonstrated that five of these--a foundry particulate and fly ash samples from three conventional combustion processes and one fluidized-bed process--had low to intermediate effects on AM. The samples collected from a copper and an aluminum smelter, however, were much more toxic than all others, and the copper smelter sample was the more toxic of the two. In the case of the two smelter samples, we found that in addition to the particles per se, cytotoxic soluble components released during incubation contributed to the total toxicity to AM.

In Vivo Tests

The next step, and a major objective of these studies, was to confirm the *in vitro* evaluation by demonstrating parallel effects in *in vivo* assessments of the inhalation hazard of these samples in the intact animal. Female CD_1 mice were exposed, as described, to aerosols of the copper smelter dust and the fluidized-bed coal fly ash (fly ash no. 2), particles that had shown very high and low cytotoxicity, respectively, *in vitro*. The means and standard deviations of aerosol mass concentration were $2033 \pm 153\ \mu g/m^3$ for the copper smelter dust and $2043 \pm 308\ \mu g/m^3$ for the fly ash. Both aerosols had log-normal size distributions, with count median diameters and σ_g's of 0.225 μm and 2.9 for the copper smelter dust and 0.134 μm and 2.1 for the fly ash.

The effects of inhalation of the particles were evaluated after 5, 10, and 20 exposures by examining changes in pulmonary cellular lavage, in susceptibility to respiratory streptococcal infection, and in pulmonary bactericidal activity to inhaled radiolabeled *K. pneumoniae*. Results are summarized in Figures 1, 2, and 3.

Total cell counts and ATP levels (expressed as percent of the control responses; see Figure 1) generally did not change significantly or exhibit any trend related to the number of aerosol exposures. Similarly, differential cell counts and percent viability of the lavaged cells (not shown) were not affected by the exposures. However, as seen in Figure 2, the percent mortality after streptococcus inhalation challenge was greater in aerosol-exposed than in control mice, for 5, 10, and 20 daily 3-h exposures to copper smelter dust aerosols ($2\ mg/m^3$). No significant changes were observed for any of the exposures to the coal fly ash. Mean survival time (not shown in the figures) was significantly lower than that of the control groups only following inhalation of the copper smelter dust (i.e., treatments that also significantly increased the mortality rate). The percent of bactericidal activity in response to inhaled radiolabeled *K. pneumoniae* was significantly less for exposed than for control mice (Figure 3), for 5, 10, or 20 daily 3-h exposures to the copper smelter dust aerosol. Similar doses of the coal fly ash aerosol had no effect.

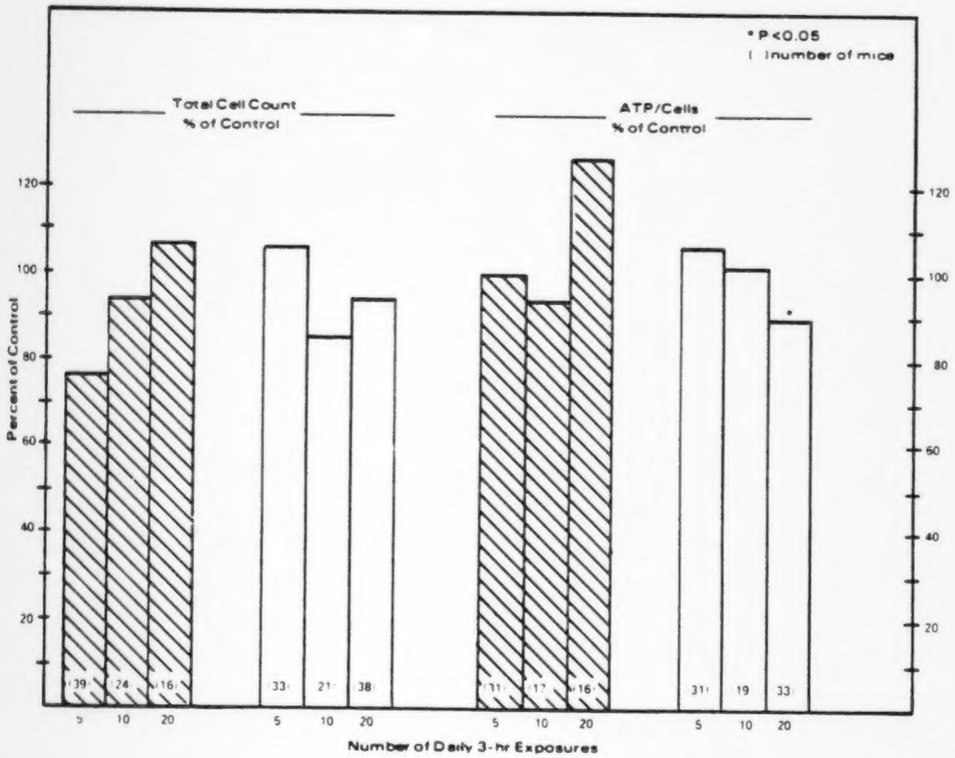

Figure 1. Changes in pulmonary cellular lavage following multiple daily 3-h aerosol exposures of mice to 2 mg/m^3 of copper smelter dust (shaded bars) or fluidized-bed coal fly ash (unshaded bars).

Thus, these data clearly demonstrate that the copper smelter dust was not only more cytotoxic in vitro to AM but also more deleterious in inhalation exposures in vivo than was the fluidized-bed coal fly ash.

CONCLUSIONS

In assays of the < 3 µm size fraction of a series of stationary point-source samples by the RAM test, generally low-to-intermediate cytotoxicity was found for samples collected from a foundry and from several coal-fired power plants. However, particulate samples from an aluminum and a copper smelter were highly toxic to AM, as measured by viability and total cellular

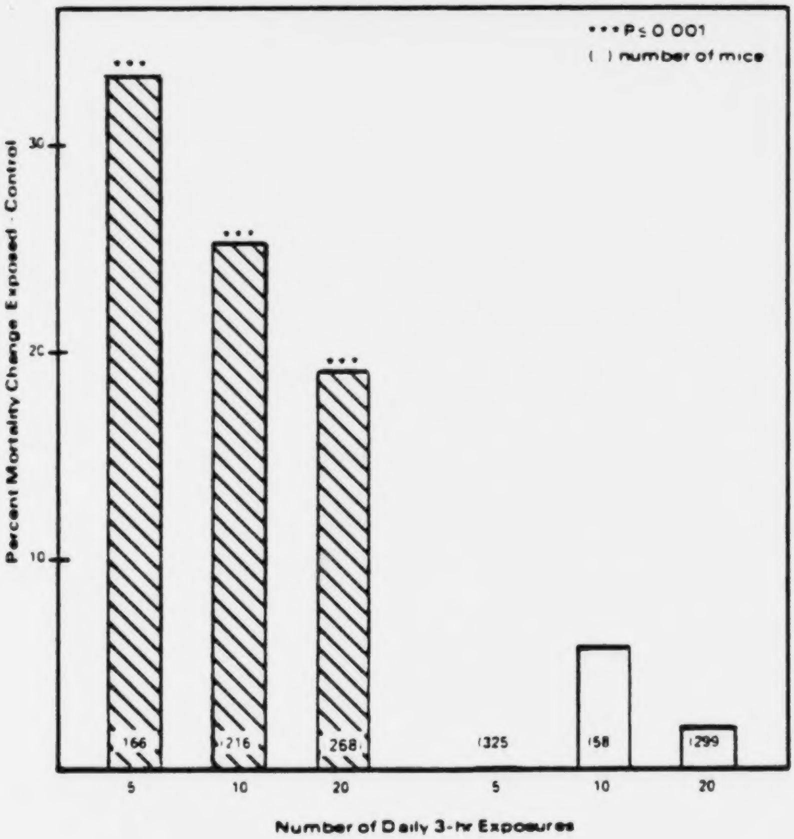

Figure 2. Excess mortality from streptococcus aerosol infection in exposed mice following multiple daily 3-h aerosol exposures to 2 mg/m^3 of copper smelter dust (shaded bars) or fluidized-bed coal fly ash (unshaded bars).

protein and ATP levels. In contrast to all others, the two smelter samples also contained soluble components that contributed substantially to their overall in vitro cytotoxicity.

The copper smelter particles and the fluidized-bed coal fly ash, chosen on the basis of their respectively high and low in vitro cytotoxicity, were used in aerosol exposures to examine their effects in vivo on pulmonary free cells, bactericidal activity, and resistance to respiratory infection in mice. The results obtained after multiple daily 3-h exposures to 2 mg/m^3 of these aerosols correlated well with the in vitro data: inhalation of the aerosols

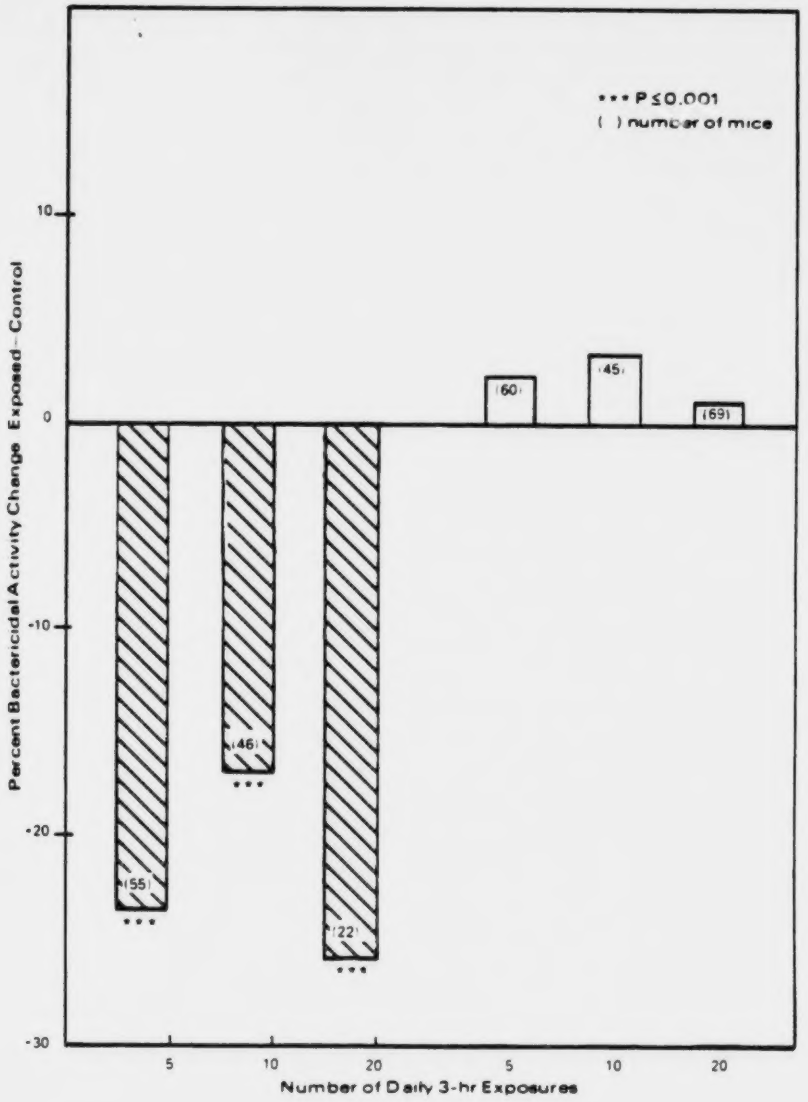

Figure 3. Percent change in bactericidal activity in response to inhaled *K. pneumoniae* in exposed mice following multiple daily 3-h aerosol exposures to 2 mg/m^3 of copper smelter dust (shaded bars) or fluidized-bed coal fly ash (unshaded area).

of the copper smelter dust produced significant differences from the controls in more of the experimental parameters than did aerosols of the coal fly ash sample. Thus, the validity of inhalation hazard prediction on the basis of an *in vitro* screening assay has been demonstrated.

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MUTAGENICITY AND CARCINOGENICITY OF A RECENTLY CHARACTERIZED CARBON BLACK ADSORBATE: CYCLOPENTA(CD) PYRENE

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INTRODUCTION

Polycyclic aromatic hydrocarbons (PAH) are widespread environmental contaminants that may be metabolically activated to mutagenic or carcinogenic derivatives (Particulate Polycyclic Organic Matter, 1972; Gelboin and Ts'o, 1978). Intensive research indicates that PAH are promutagens or procarcinogens containing the bay region geometric feature (Jerina et al., 1978). Studies show that the biological activity of these compounds results from metabolism to bay region diol-epoxides, which are capable of forming covalent adducts at nucleophilic sites within DNA.

Cyclopenta(cd)pyrene (CPP, I)(see Figure 1), a non-bay region PAH, was recently characterized and shown to be highly mutagenic in the Salmonella typhimurium assay (Eisenstadt and Gold, 1978). CPP was initially identified in extracts of furnace black as the major contributor to the high mutagenic activity of the extracts. Because of its unique structure and wide environmental distribution as a component of soots (Lee et al., 1977; Grimmer, 1977; Kaden et al., 1979), carbon blacks (Wallcave et al., 1975; Gold, 1975), and cigarette smoke (Snook et al., 1977), CPP has evoked considerable interest (Ittah and Jerina, 1978; Konieczny and Harvey, 1979; Ruehle et al., 1979). The 3,4-oxide of CPP, predicted as an ultimate mutagenic metabolite, has been synthesized and shown to be a powerful direct-acting mutagen to S. typhimurium. Confirmation of the 3,4-oxide as a primary metabolite and ultimate mutagen requires that the expected enzymatic hydration product, trans CCP 3,4-dihydrodiol, be identified as a metabolic product.

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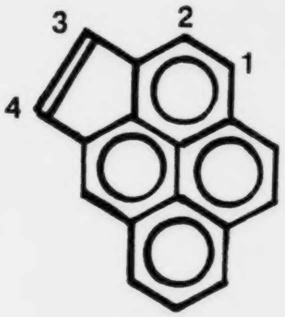

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Figure 1. Structure of cyclopenta(cd)pyrene (CPP, I).

Response of different assay systems to treatment with specific mutagens may vary (Maher et al., 1978), and for this reason, the mutagenicity of CPP and its 3,4-oxide in mammalian cells as well as in microbial systems is of interest.

This study reports identification of trans-CPP 3,4-dihydrodiol as the major CPP metabolite of both 3-methylcholanthrene- (3-MC) and Aroclor-1254-induced rat liver microsomes. CPP and its 3,4-oxide were tested for mutagenicity in the L5178Y mouse lymphoma system and for cell transformation in the C3H10T1/2CL8 mouse embryo fibroblast system. The results are discussed in relation to the proposal that CPP 3,4-oxide may be an ultimate mutagenic or carcinogenic metabolite of CPP.

MATERIALS AND METHODS

Chemicals

CPP, CPP 3,4-oxide, and 4-oxo-CPP were obtained as previously described (Gold et al., 1978, 1979). Tritiated CPP ([H^3]CCP) was prepared by catalytic exchange labeling (New England Nuclear, Boston, MA) and was purified prior to use by chromatography over silica.

Carbon Black Analysis

Semireinforcing furnace black was extracted with methylene chloride (CH_2Cl_2) and fractionated by standard procedures (Gold, 1975; Rosen and Middleton, 1955). A 120-mg aliquot of the PAH fraction was further separated on a size B Lo Bar silica column (Merck) with an initial eluent of 4% CH_2Cl_2 in hexane changed to 5% CH_2Cl_2 in hexane after 500 ml. The separation was followed by ultraviolet (UV) detection, and individual peaks were collected.

Ames assays were performed with strain TA100 as described by Ames et al. (1975). Dose-response curves were obtained from 1, 2, 4, 10, and 20 μg of test mixture per plate, and mutagenic potency (revertants per microgram) was determined from the slope of the linear portion of the curve. Metabolic activation was supplied by 0.5 ml S-9 from Aroclor-treated rats.

Metabolism Studies

Rat liver microsomes and S-9 were prepared from Aroclor-1254-treated male Sprague-Dawley rats weighing 150 to 200 g, according to the procedures of Ames et al. (1975), except that the microsomal pellet was resuspended in buffer and centrifuged a second time at 100,000 x g for 60 min. Microsomes were stored frozen at -80°C until used.

The concentrations of ingredients in the 1-ml microsomal metabolism mixture were: 50 mM Tris-hydrochloride, pH 7.4; 3 mM magnesium chloride ($MgCl_2$); 0.8 mM NADP; 5 mM glucose-6-phosphate; 0.4 units glucose-6-phosphate dehydrogenase; 0.89 mM [G-^{3}H]CPP (specific activity, 5 x 10^4 dpm/nmol) and 0.2 mg microsomal protein. The reaction was started by first adding [^{3}H]CPP to the microsomal mixture at 4°C and then shaking the mixture at 37°C for the indicated times. One volume of acetone was added to stop the reaction. Two volumes of ethyl acetate were added and the mixture was shaken or vortexed vigorously. The ethyl acetate-acetone phase was removed, dried with anhydrous sodium sulfate (Na_2SO_4) and evaporated to dryness under nitrogen gas. The residue was dissolved in a small volume of methanol and subjected to high performance liquid chromatography (HPLC) analysis. Ninety to ninety-five percent of the input radioactivity was recovered in the organic extract.

Aroclor-1254- and 3-MC-induced enzymes produced similar metabolite profiles (Figure 2), and preparative work was done with 3-MC-induced S-9 activation using 50- to 100-ml reaction volumes.

HPLC separations were performed on a Perkin-Elmer Series II liquid chromatograph with a Perkin-Elmer 4.6-mm x 25-cm ODS-SilX-I column or 2.6-mm x 25-cm ODS SilX-II column. A UV detector at 254 nm was used to monitor fractions for preparative work. Fractions were collected (0.5 ml) and counted by liquid scintillation for quantitative analysis of [^{3}H]CPP metabolites.

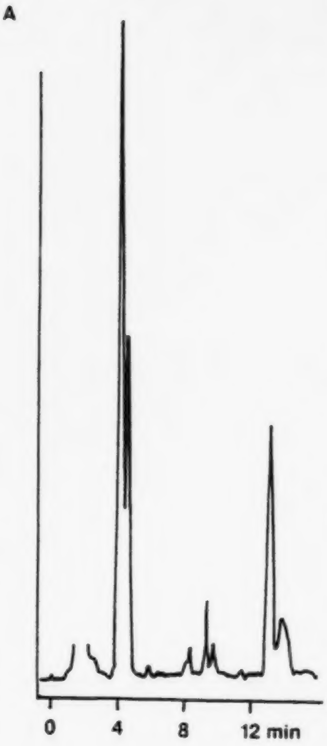

Figure 2. HPLC trace at 254 nm of CPP metabolites from (A) Aroclor-1254-induced rat liver microsomes (ODS SilX-II column, 2.6 mm x 25 cm) and (B) 3-MC-induced rat liver microsomes (ODS SilX-I column, 4.6 mm x 25 cm).

The same linear gradient was used for all separations: a 1%/min gradient from 35:65 H_2O:methanol to 100% methanol, at a flow rate of 1.5 ml/min.

In Vitro Mammalian Mutagenesis Assay

The $TK^{+/-}$L5178Y mouse lymphoma mutagen assay, developed by Clive and co-workers to identify mutagens that induce genetic damage at the thymidine kinase (TK) locus, was performed according to published methods (Clive et al., 1979; Clive and Spector, 1975), using Fischer's medium. Trifluorothymidine (1 µg/ml) was used to select for thymidine-kinase-deficient mutants. CPP, dissolved in to published methods (Clive et al., 1979; Clive and Spector, 1975), using Fischer's medium. Trifluorothymidine (1 μg/ml) was used to select for thymidine-kinase-deficient mutants. CPP, dissolved in dimethylsulfoxide (DMSO), was tested in 3% horse serum at a cell concentration of 0.6 x 10^6 cells/ml, with and without an Aroclor-1254-induced rat hepatic S-9 activation system. The CPP 3,4-oxide (dissolved in DMSO) was tested in 10% horse serum at a cell concentration of 0.6 x 10^6 cells/ml without metabolic activation. The positive controls ethyl methanesulfonate (EMS) and 2-acetylaminofluorene (AAF) were dissolved in saline and DMSO, respectively. Two days were allowed for the expression of newly induced mutants.

Oncogenic Transformation Assay

The mouse embryo fibroblast cell line C3H10T1/2CL8 was derived by Reznikoff et al. (1973; Nesnow and Heidelberger, 1976; Gehly et al., 1979) and donated by Dr. Charles Heidelberger for use in these experiments. Cell cultures were incubated at 37°C in humidified incubators with an atmosphere of 5% carbon dioxide in air. All cultures were grown in Eagle's Basal Medium with Earle's salts and L-glutamine supplemented with 10% heat-inactivated fetal calf serum (Grand Island Biological Co.). The cells were routinely checked for *Mycoplasma* contamination and found to be *Mycoplasma*-free.

For transformation, cells were seeded onto 60-mm petri dishes (1000/dish) in 5 ml of medium (12 replicates/treatment) and 24 h later treated with the hydrocarbon dissolved in acetone (25 μl). After an additional 24 h, the medium was removed, and the cells received fresh complete medium containing penicillin (100 units/ml) and streptomycin (50 μg/ml). Medium was changed weekly until the cells reached confluency, whereupon the fetal calf serum concentration was reduced to 5%.

At the end of six weeks, the dishes were washed with 0.9% sodium chloride solution, fixed with methanol, stained with Giemsa, and scored for oncogenic transformation. Three different types of foci have been described after treatment of C3H10T1/2CL8 cells with polycyclic hydrocarbons. Only Type II and Type III foci were scored in this assay, since it has been demonstrated that these foci produce fibrosarcomas upon injection into irradiated C3H mice, 33% and 67% of the time, respectively.

Control cultures received the appropriate solvent and were treated the same way as the exposed cells. Cytotoxicity assays were performed concurrently with the transformation assays, and using the same protocol, except that the dishes were plated with 200 cells (6 replicates/treatment) and stained 10 to 12 days later.

RESULTS AND DISCUSSION

A fractionation scheme was applied to the organic extract of semireinforcing furnace black used in the rubber industry. The Ames test was used in conjuction with the chemical separation to identify mutagens. The data shown in Table 1 indicate that the PAH fraction of the CH_2Cl_2 extract of the semireinforcing furnace black accounted for essentially all of the mutagenicity in the total extract. Table 2 shows the mutagenic activity of the fractions obtained on further chromatographic resolution of the PAH fraction. Specific activity of the total PAH fraction calculated from the activities of the fractions resolved in Table 2 appeared to be about the same as that observed for the total PAH fraction (see Table 1). A similar observation has been reported for kerosene soot (Kaden et al., 1979). It is apparent that benzo(a)pyrene (B[a]P) contributed only slightly to the mutagenic activity of the PAH fraction and therefore to the mutagenic activity of the total organic extract, while CPP was the compound principally responsible for the activity. An estimate of mutagenicity based on B(a)P content would have seriously underestimated the mutagenicity of the sample. This result underscores the inherent danger in using a marker compound such as B(a)P as an index of hazard even for samples with similar pyrogenic origins like soots and carbon blacks.

Table 1. Mutagenic Activity of Fractionated Carbon Black Extracts in *Salmonella* Strain TA100

Fraction	Weight (mg)	Activity (rev/μg)
Neutral		
saturated hydrocarbon	9	not active
polycyclic aromatic	135	38
polar	27	4
Acidic	49	0.5[a]
Basic	6	not active
Total Extract[b]	346	14

[a] Nonlinear dose-response curve.

[b] From 314 g furnace black.

Table 2. Distribution of Mutagenic Activity in Aromatic Fraction of Furnace Black Extract with Ames Strain TA100

Fraction Number	Composition	Fraction Weight (mg)	Activity (rev/µg)	Contribution to Total Polycyclic Aromatic Activity (% wt. of Polycyclic Aromatic Fraction x Activity)
1	naphthalene acenaphthylene phenanthrene	10.4	not active	
2	pyrene	70	not active	
3	fluoranthene benzo(ghi)fluoranthene[a] cyclopenta(cd)pyrene	30	93	21
4	benzo(ghi)fluoranthene cyclopenta(cd)pyrene[a]	8.4	380	24
5	cyclopenta(cd)pyrene isomers of molec. wt. 252	2	90	1
6	benzo(a)pyrene benzo(e)pyrene benzo(ghi)perylene isomers of molec. wt. 276 and 300	4.6	29	1
Calculated activity of total polycyclic aromatic fraction (rev/µg)				47

[a] Major component.

Structure-reactivity relationships for CPP were of considerable interest because of its high mutagenicity and unusual structure--containing a fused cyclopenteno ring and lacking a bay region. Hence, an investigation of the metabolism, mutagenicity, and carcinogenicity of CPP was undertaken.

To identify the structure of the metabolic products of CPP, synthetic metabolites were produced by acid-catalyzed decomposition of the 3,4-oxide. This procedure cleanly yielded three products (Figure 3). The two most polar peaks were identified as the 3,4-dihydrodiol isomers based on the mass spectrum and UV spectrum of the mixture. As a result of the rigid planarity of the fused five-membered ring in CPP, the trans diol was expected to be the less polar isomer, because the dipole moments of the hydroxy substituents are largely opposing. The rigid geometry also led to the prediction of greater shielding for the C^3 and C^4 protons of the trans isomer in the nuclear magnetic resonance (NMR) spectrum. The major diol resulting from hydration was the less polar isomer (peak B, Figure 3) and had the more shielded C^3 and C^4 protons in the NMR spectrum (Figure 4a). On this basis, it was assigned the trans configuration. This assignment was consistant with the expectation that the major isomer of acid-catalyzed epoxide hydration would be the trans isomer (Bruice et al., 1976; Keller and Heidelberger, 1976).

Based on its physical-chemical properties, the third and major product of the acid catalyzed reaction was identified as 4-oxo CPP. As illustrated in Figure 5, it was readily distinguished by UV spectroscopy from the known 3-oxo compound (Gold et al., 1978).

The HPLC chromatograms (Figure 2) of the metabolic mixtures produced by 3-MC- and Aroclor-1254-induced rat liver microsomes indicated a single major metabolite accounting for 50% of the products. This metabolite was identified as trans-CPP 3,4-dihydrodiol. Its chromatographic retention time and NMR spectrum (Figure 4b) corresponded to those of the trans isomer from the hydration of the 3,4-oxide. Also, its UV and mass spectrum were consistent with the assigned structure.

The $TK^{+/-}$ L5178Y mouse lymphoma assay has been used to measure the mutagenicity of diverse chemical agents (Clive et al., 1979). Since these cells lack the enzymes necessary to activate promutagens, CPP was tested both with and without S-9 activation, to confirm the activation-dependence of mutagenicity typical of PAH. As expected, CPP was not mutagenic without activation over the concentrations tested (0.75 to 30 μg/ml)(unpublished data). On activation by Aroclor-1254-induced hepatic S-9, CPP was mutagenic, with twice the mutation frequency of the control (Table 3). The 3,4-oxide was tested without activation to determine

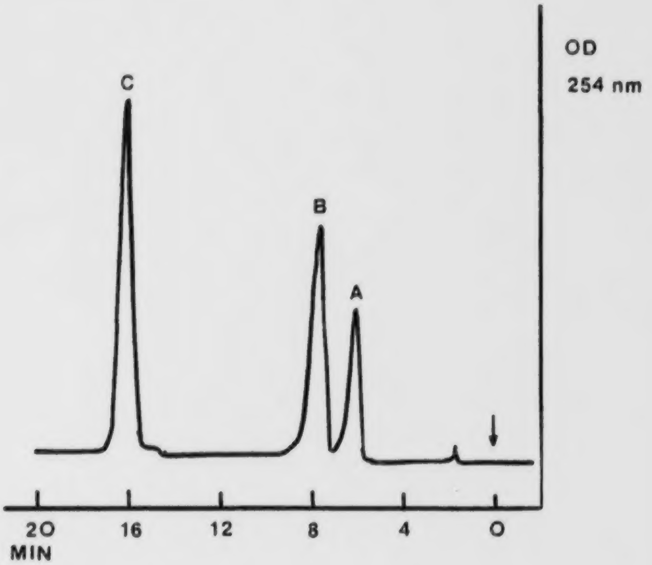

Figure 3. HPLC trace at 254 nm of acid catalyzed decomposition products of CPP 3,4-oxide, peak A, cis-CPP 3,4-dihydrodiol; peak B, trans-CPP 3,4-dihydrodiol; peak C, 4-oxo CPP.

whether it was a direct-acting (and a possible ultimate) mutagen. It was found to be mutagenic to L5178Y cells over a dose range similar to that of CPP. Over the 70 to 20% survival range, the oxide was two- to six-fold more mutagenic than the parent hydrocarbons.

The C3H10T1/2CL8 transformation assay responds to a variety of carcinogens, including PAH (Nesnow and Heidelberger, 1976). C3H10T1/2CL8 mouse embryo cells contain cytochrome P-450 mixed function oxidase, epoxide hydrase, and conjugating enzymes necessary to metabolize and activate or detoxify chemical carcinogens, especially PAH (Gehly et al, 1979; Nesnow and Heidelberger, 1976). As shown in Table 2, CPP produced a dose-related response in the formation of both Type II and Type III transformed foci. At the highest dose used, every plate contained at least one Type III focus. In accord with the recent report (Wood et al., 1980) that CPP is a weaker tumor initiator than B(a)P, the data in Table 4 indicate that CPP was also less active than B(a)P in the C3H10T1/2CL8 transformation assay.

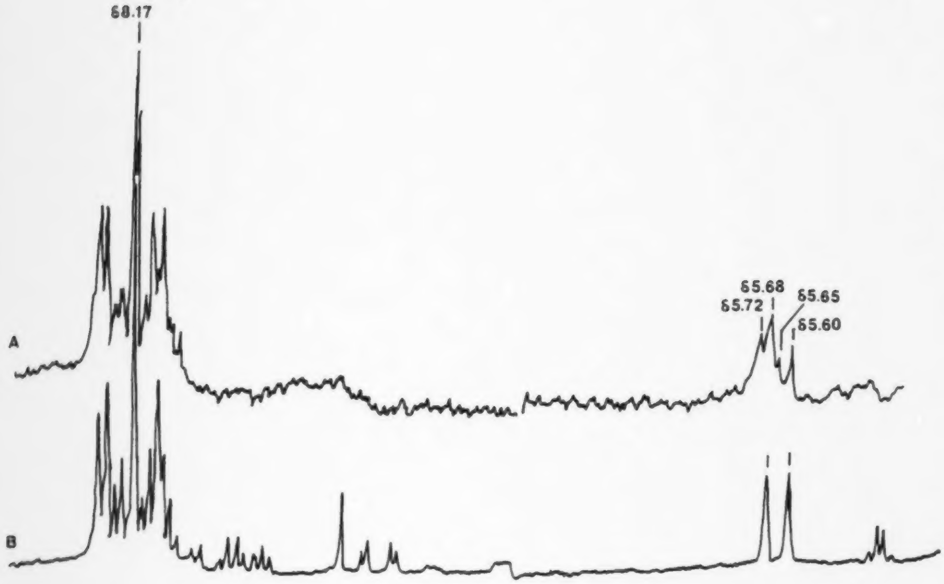

Figure 4. In part a, 270 MHz NMR (acetone-d_6) of *cis*-*trans*-CPP 3,4-dihydrodiol mixture. In part b, 270 MHz NMR (acetone-d_6) of major CPP metabolite. Underlined resonances are consistent with CPP 9.0-dihydrodiol cochromatographing with the 3,4-dihydrodiol.

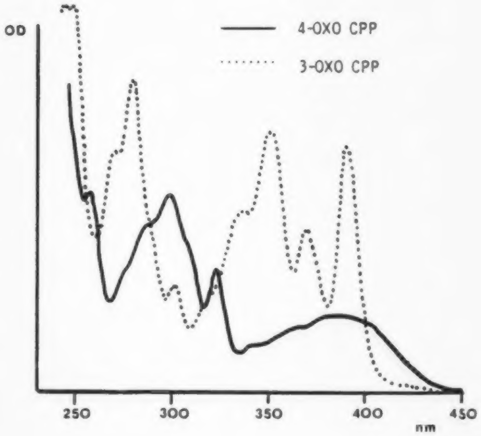

Figure 5. UV-VIS spectrum (CH_2Cl_2) of 4-oxo and 3-oxo CPP.

Table 3. Mutagenesis of $TK^{+/-}$ L5178Y Mouse Lymphoma Cells by CPP and CPP 3,4-Oxide

Concentration (µg/ml)	Total Viable Clones	Total Mutant Clones	Total Survival[a] (% of Control)	Mutant Frequency (x 10^6)
CPP (with S-9)				
1.20	439	263	110	120
1.30	365	317	74	172
1.40	436	300	56	138
1.50	407	284	44	139
1.60	399	375	20	188
2-AAF				
30	270	785	31	581
DMSO (1%)	453	178	100	79
CPP 3,4-oxide				
0.60	536	783	71	292
0.70	472	803	63	340
0.84	536	912	72	340
0.96	463	776	58	335
1.08	475	1234	52	519
1.20	476	1065	44	448
1.32	406	1174	32	578
1.44	341	1090	22	640
1.56	360	1423	23	790
1.68	459	1118	36	487
1.80	273	1204	10	882
2.04	333[b]	1214	6	1460
EMS				
500	259	1638	25	1267
DMSO (1%)	623	297	100	95
Untreated Control	558	220	100	79

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[a] Survival calculations described by Clive and Spector (1975) combine both relative growth in suspension and relative plating efficiency.

[b] Cells cloned at a density of 12 cells/ml; all other cultures cloned at 6 cells/ml to determine viability.

Table 4. Morphological Transformation of C3H10T1/2CL8 Cells by CPP and CPP 3,4-Oxide

Concentration (μg/ml)	Plating Efficiency (%)	No. Type II Foci /Total Dishes	No. Type III Foci /Total Dishes	Dishes with Type II and III Foci (%)
CPP				
0.01	27	0/12	2/12	17
0.03	28	1/12	1/12	17
0.1	26	0/12	1/12	8
.3	27	3/12	2/12	25
1.0	29	4/11	7/11	73
3.0	29	6/12	7/12	75
10.0	22	13/12	12/12	100
CPP 3,4-oxide				
0.001	32	2/12	0/12	8
0.003	31	1/11	0/11	9
0.01	30	0/12	0/12	0
0.03	32	1/11	0/11	9
0.1	32	1/12	0/12	8
0.3	32	0/12	2/12	17
1.0	31	0/12	2/12	17
3.0	27	1/12	2/12	25
Acetone (0.5%)[a]	30	2/24	0/24	8
B(a)P[a] (1)	18	38/24	59/24	100

[a] Results from two separate experiments combined.

CPP 3,4-oxide transformed C3H10T1/2CL8 cells at concentrations of 0.3 μg/ml. At 3 μg/ml, it produced both Type II and Type III foci, with virtually no toxicity. The lack of toxicity is remarkable, because structurally related K-region oxides of B(a)P and 3-MC were highly cytotoxic (unpublished data). Although the direct-acting oxide was a more potent mutagen than CPP, it was less active than the parent hydrocarbon in the C3H10T1/2CL8 transformation assay. This was probably due to the C3H10T1/2CL8 cells' ability to detoxify arene oxides to dihydrodiols (Nesnow and Heidelberger, 1976; Gehly et al., 1979). L5178Y mouse lymphoma cells lack this ability (Clive et al., 1979).

The potent direct-acting mutagenicity of the 3,4-oxide in both bacterial and mammalian assays, its ability to transform C3H10T1/2CL8 cells, and the identification of CPP 3,4-dihydrodiol as a major metabolite (Gold et al., 1979) in 3-MC- and Aroclor-induced metabolism of CPP are strong evidence that CPP 3,4-oxide is both a primary product of enzymatic oxidation and an ultimate mutagen or carcinogen. Further support for this conclusion is found in the report that the addition of epoxide hydrase to a purified, reconstituted monooxygenase activating system drastically reduces the mutagenicity of CPP (Wood et al., 1980). The effect of epoxide hydrase can readily be explained if CPP 3,4-oxide is the ultimate mutagen and, like other arene oxides (Gelboin and Ts'o, 1978), is a good substrate for epoxide hydrase. Since the B(a)P bay region diol-epoxides are poor substrates for epoxide hydrase (Gelboin and Ts'o, 1978), CPP would be less mutagenic than B(a)P in the C3H10T1/2CL8 system due to the more rapid deactivation of metabolically-generated CPP 3,4-oxide.

An important distinction between B(a)P diol-epoxides and CPP 3,4-oxide is that the latter is an arene oxide: the epoxidized bond is adjacent to the aromatic nucleus at both termini. The arene oxides tested, 3-MC 11,12-oxide and B(a)P 4,5-oxide, both failed to transform C3H10T1/2CL8 cells (unpublished data). CPP is the first arene oxide reported to transform this cell type.

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MUTAGENICITY OF COAL GASIFICATION AND LIQUEFACTION PRODUCTS

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INTRODUCTION

As it becomes evident that the shortage of oil resources is not a transient phenomenon, emphasis is being directed to the use of domestic coal. Increased use of coal, however, adds coal combustion products to the pollution burden. Although various technologies are being developed to produce cleaner-burning fuels from coal, such as gaseous fuels, de-ashed low-sulfur boiler fuels, and synthetic crude oils, problems remain in the production of these materials.

The production of liquid fuels from coal has been associated with an increased risk of cancer, and certain of these coal-derived liquids have been shown to be carcinogenic in experimental animals (Bingham, 1975; Ketcham et al., 1960; Sexton, 1960a, b; Weil et al., 1960). Heavy exposure to coal hydrogenation materials causes both benign and malignant skin tumors. Composition data (Swansiger, 1974; Battelle, 1974; ORNL, 1975; Electric Power Res. Inst., 1975; ERDA, 1976) suggest that various liquefaction products and by-products are likely to contain polycyclic substances of considerable carcinogenic potential; these compounds are most likely to be found in the high-boiling-point aromatic fractions of the product liquids (TWR, 1976). The total products of hydrogenation, high-boiling-point distillates, centrifuged oils, char, residues, recycled solvent oil, recycled solvent, and liquid coal are all potentially hazardous materials (Freudenthal et al., 1975).

Synthetic natural gas is not expected to pose a carcinogenic risk, as any trace elements, organic carcinogens, or cocarcinogens present in the raw product will have been removed during clean-up and scrubbing operations. Rather than the fuel produced, it is the coal gasification process itself that should be the primary concern (Kornreich, 1976). The greatest potential hazard in coal gasification is in the early stages of the processes, in which coal goes through a series of structural degradations of complex organic compounds. During these early stages, leaks and spills should contain hazardous material (Freudenthal et al., 1975). Potentially carcinogenic polycyclic organic material is likely to concentrate in the tars, oils, and char (Kornreich, 1976; TRW, 1976). The crude gas, if it contains tars of high-boiling-point oils, must also be considered a potential hazard.

Hazardous chemicals may be synthesized whenever coal is subjected to severe conditions such of those of pyrolysis, hydrogenation, or gasification. As use of synthetic fuels will probably increase, sensitive and rapid *in vitro* studies should be carried out on products, intermediate streams, and wastes of coal conversion processes to determine potential hazards. A variety of techniques are available for assessing the environmental risks and potential health effects of coal processing technologies. These techniques include chemical and physical characterizations, microbial assays, and tests for acute toxicity and irritation, subchronic toxicity and teratology, chronic toxicity, and carcinogenesis, among others. To conduct so many tests on all new technological developments would not be appropriate, as each change of experimental condition or operation would lead to new health study requirements. The cost would be prohibitive, and much of the resulting data useless, as it would apply to defunct processes. Delaying biological assessment until the process is ready for production would likewise be inappropriate. An acceptable position on testing new fossil energy processes must be found. A suggested compromise would involve chemical characterizations and rapid bioassay studies in small-scale developmental programs followed by detailed characterization and short-term and long-term toxicological testing programs on pilot processes.

For this project, materials ranging from solid residue to liquid products and waters, produced through advanced coal-conversion technologies (including gasification and liquefaction), have been selected and screened using the *Salmonella*/microsomal mutagenesis assay. This assay, developed by B.N. Ames et al. (1975), is recognized as one of the most useful short-term assays for mutagenesis, based on the number of compounds it detects as mutagens and on its high correlative of positive responses with known carcinogens (Bridges, 1976; McCann et al., 1975; McCann and Ames, 1976; Purchase et al., 1976; Simmon 1979; Committee 17, 1975). It is being used to investigate the health effects of compounds already in the environment and of materials under development. It is particularly useful in the evaluation of mixtures of substances, such as cigarette smoke concentrate (Kier et al., 1974; Sato et al., 1977), synthetic crude oil (Epler, 1978; Epler et al., in press), and organic extracts of drinking water (Loper et al., 1978).

MATERIALS AND METHODS

Sample Preparation

Coal-related materials were provided by the Department of Energy. These samples are not considered to be process discharges and may not be identical to materials eventually generated from advanced coal processes developed for commercial use. Although the samples are not representative of all materials derived from advanced coal processes, they were selected for study because of their immediate availability and the desirability of testing materials of widely differing properties.

All samples were stored at 5°C. Materials ETTM-01, ETTM-02, ETTM-08, and ETTM-09, which were tars or viscous liquid, were prepared for testing by weighing a small amount (20 to 70 mg) and adding dimethylsulfoxide (DMSO) to obtain a presumptive concentration of 10 mg/ml. In no case did all the material dissolve. The amount of insoluble sample was subtracted from the total to give the concentration used in calculating the mutagenic doses. The sample solutions were filter-sterilized prior to testing. All samples were applied in 0.1-ml aliquots. The sample solutions were further diluted in DMSO, so that the following percentages of the sample solution were assayed: 100%, 50%, and 0.5%, or 100%, 50%, 10%, 5%, and 1%. For ETTM-02, the liquid fraction was assayed by applying 0.1 ml of the undiluted substance, as well as the concentrations listed above.

The powdered samples ETTM-03 and ETTM-04 did not dissolve in DMSO, nor were mutagenic substances in detectable quantities washed from them into the DMSO. Aqueous leachates were made from these samples. Five grams of the powder were added to 45 g distilled water at pH 3.0, 5.0, 6.0, 7.0, and 10.0. These suspensions were stoppered and shaken at room temperature overnight. Each suspension was centrifuged, and the supernatant fraction was aspirated and filter-sterilized for use in the mutagenic assays.

Liquids ETTM-06 and ETTM-07 changed from yellow to turquoise when mixed with DMSO, and ETTM-06 underwent an exothermic reaction. These two samples were also diluted in double-distilled water. The black particulate matter floating on sample ETTM-06 was removed during filter-sterilization, and the solvent and sample phases were mixed vigorously immediately prior to assay.

Organic extracts of ETTM-01, ETTM-02, ETTM-08, and ETTM-09 were prepared as follows: Approximately 5 g or less of each sample was weighed, and a volume (in ml) of solvent equal to five times the sample weight (in g) was added. This mixture was agitated vigorously in the dark at room temperature for two hours. After centrifugation to settle particulates, the solvent was removed, and an equal amount of fresh solvent was added. The two extracts were pooled and evaporated under nitrogen gas. This procedure was carried out sequentially with hexane, toluene, methylene chloride, and acetonitrile. This simple organic extraction procedure was chosen for its ease and appropriateness for these samples. The type of organic extracts produced, moreover, is suitable for analysis by high performance liquid chromatography.

Mutagenicity Assays

Salmonella/microsomal assays were carried out according to the methods described by Ames et al. (1975). Microsomal extracts for routine assays (S-9) were made from livers of male Sprague-Dawley rats (150 to 200 g body weight) that had been administered 500 mg/kg Aroclor 1254 (PCB) on day one and killed on day six. On the day of assay, S-9 from four animals was pooled. Assays were also done using S-9 from rats treated with 3-methylcholanthrene (3-MC, 40 mg/kg) and from rats given corn oil. Plates were scored using an automatic colony counter. Only counts of at least twice the spontaneous values were considered to indicate mutagenicity. Colony counts below the range observed for spontaneous revertants, clearing of bacterial lawns, or the appearance of pinpoint *his*$^-$ colonies was scored as a toxic response.

RESULTS AND DISCUSSION

Table 1 summarizes the mutagenic activity of the samples. No sample was direct acting (i.e., mutagenic in the absence of S-9). The liquefaction products, distillate oils, heavy liquid, and residue were mutagenic. Of the gasification products, only the tar was active in these assays. Neither DMSO extracts nor aqueous leachates of gasification particulate and ash were mutagenic.

Figure 1 is a plot of dose-response data for ETTM-01. This plot, typical of those for the other active samples, indicates a linear dose response at the lowest concentrations tested, with decreases in the slopes of the curves at the higher concentrations.

Table 1. Mutagenicity of Dimethylsulfoxide-soluble Components of Coal-related Materials

Sample		Mutagenicity[a] TA1535	TA1538	TA98	TA100
ETTM-01	Liquefaction product	-	+	+	+
ETTM-02	Gasification tar	-	+	+	+
ETTM-03	Gasification particulate	ND	ND	-	-
ETTM-04	Gasification ash	ND	ND	-	-
ETTM-06	Liquefaction untreated water	ND	ND	-	-
ETTM-07	Liquefaction light oils	ND	ND	-	-
ETTM-08	Liquefaction heavy liquid (with solids)	ND	ND	+	+
ETTM-09	Liquefaction product (filtered)	ND	ND	+	+
ETTM-10	Liquefaction distillate oils	-	+	+	+
ETTM-11	Liquefaction residual	-	+	+	+

[a] + = mutagenic; - = not mutagenic; ND = not determined.

As these samples are mixtures, there are several possible explanations for this pattern:

1) The samples may be toxic to the organisms at the higher doses. When these concentrations of sample were tested without S-9, there was apparent toxicity.

2) At the high concentrations, activation enzymes may be saturated, or metabolism channelled into detoxification pathways.

3) Components in the mixture may interact, contributing to nonlinear kinetics.

Revertant colonies per microgram of sample, derived through regression analysis of the linear portion of the dose response, are given in Table 2. Strains TA98 and TA1538 (frameshift mutants with and without a misrepair-enhancing plasmid) were the most sensitive to the mutagenic action of the samples. The amount of Aroclor-induced S-9 routinely used in the assay was 50 µl/plate. Figure 2 is a representative plot of mutagnenicity in response to varying

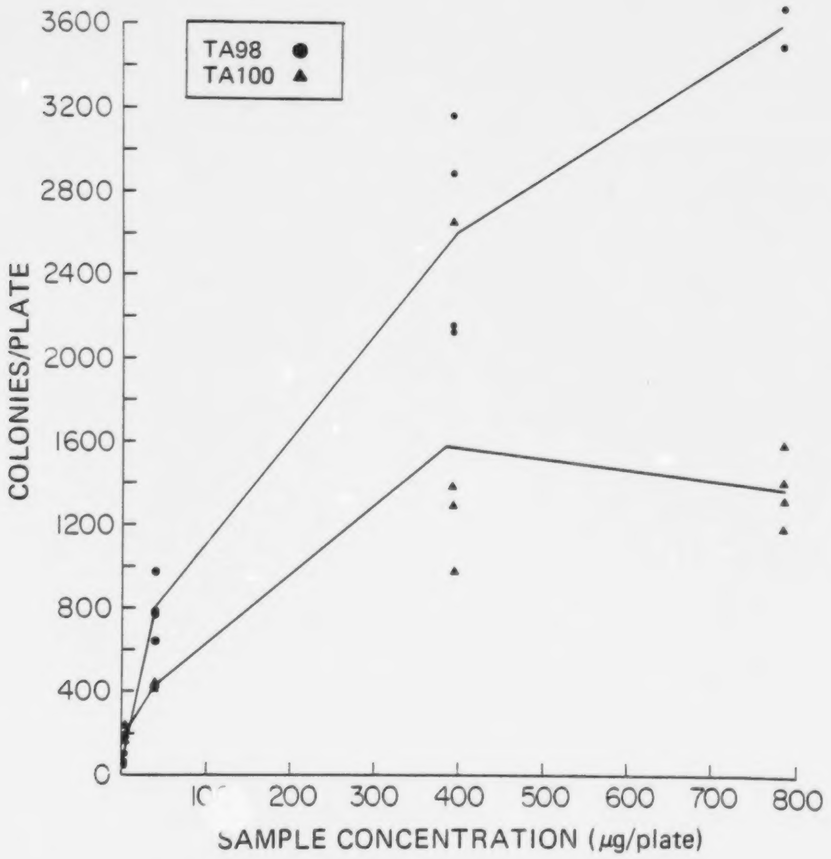

Figure 1. Mutagenicity of ETTM-01 in Salmonella with Aroclor-induced S-9 (50 μl/plate). Correlation coefficients (r) for the linear regression lines up to 39.3 μg of sample are as follows: strain TA98, 0.982; TA100, 0.971. For concentrations above 39.3 μg/plate, lines are drawn through the means of the data points.

amounts of S-9. For all the mutagenic samples, 50 μl of S-9 provided optimal or nearly optimal activation for mutagenesis. The active samples were also assayed for mutagenicity in the presence of 50 μl/plate uninduced S-9 and 50 μl/plate 3-MC-induced S-9. Uninduced S-9 was uniformly poor for sample activation. For all but one sample (ETTM-01; see Figure 3), Aroclor-induced S-9 was most effective in generating metabolites mutagenic for TA98 and TA100.

Table 2. Relative Mutagenic Activities of Coal-related Materials

A.

Sample	Revertant Colonies/μg[a] TA1535	TA1538	TA98	TA100
ETTM-01	-	30.12	18.54	6.89
ETTM-02	-	11.16	6.75	6.49
ETTM-08	ND	ND	16.66	8.38
ETTM-09	ND	ND	11.55	6.76
ETTM-10	-	7.30	10.88	2.10
ETTM-11	-	27.92	26.01	11.28

B.

Coefficients of Linear Correlation (R)

	TA1358		TA98		TA100	
Sample	N[b]	R	N	R	N	R
ETTM-01	16	0.921	32	0.888	59	0.918
ETTM-02	6	0.999	46	0.918	45	0.857
ETTM-08		-	60	0.960	57	0.843
ETTM-09		-	39	0.948	54	0.887
ETTM-10	18	0.987	64	0.905	78	0.911
ETTM-11	28	0.912	64	0.933	65	0.950

[a] Calculated from linear postions of dose-response curves: - = no dose response; ND = not determined.

[b] N = number of data points.

Sequential organic extracts were prepared on two occasions from each of the six mutagenic samples. Whenever possible, five-point dose-response assays were done with strains TA98 and TA100. For comparison, a dose-response assay of the unfractionated whole sample was run in tandem with these assays. Representative results are presented in Tables 3 and 4 and Figures 4 and 5. No extracted material was mutagenic in the absence of S-9. The samples varied widely in the percentage extractable by the solvents and in the mutagenicity of the extracted fractions. For the majority of samples, the toluene-extractable components were most mutagenic, although they were not always the largest fractions by

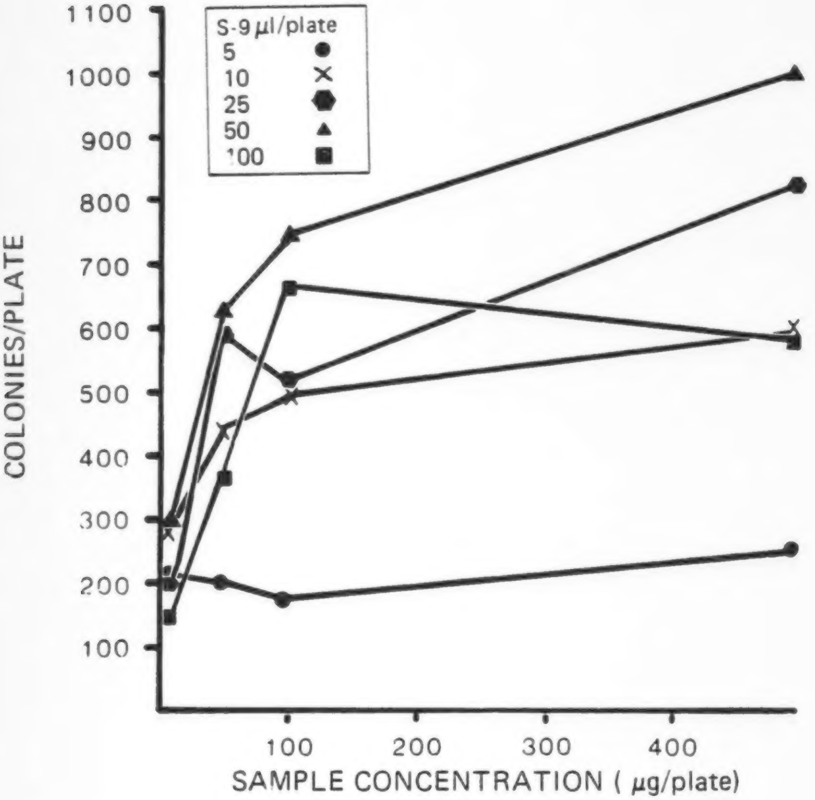

Figure 2. Effect of varying S-9 concentration on TA98 mutagenesis by ETTM-10 with Aroclor (PCB)-induced S-9.

weight. Tables 3 and 4 show the mutagenic contribution of each fraction to the whole sample (i.e., percent of whole sample present in fraction x revertant colonies per milligram). Comparing the sums of the fractional mutagenic contributions with the activity of the unfractionated sample reveals two patterns. In the first, typified by ETTM-01, the sum of the activities of the fractions was less than the activity of the whole sample. Synergistic actions among components of the sample could contribute to the greater mutagenicity of the unfractionated sample. It is also possible that material was lost or altered during extraction. ETTM-10 represents the second pattern, in which the sum of fraction activities was greater than the activity of the parent mixture. This probably indicates that antagonistic components in the whole sample inhibit total mutagenicity. It is also conceivable that

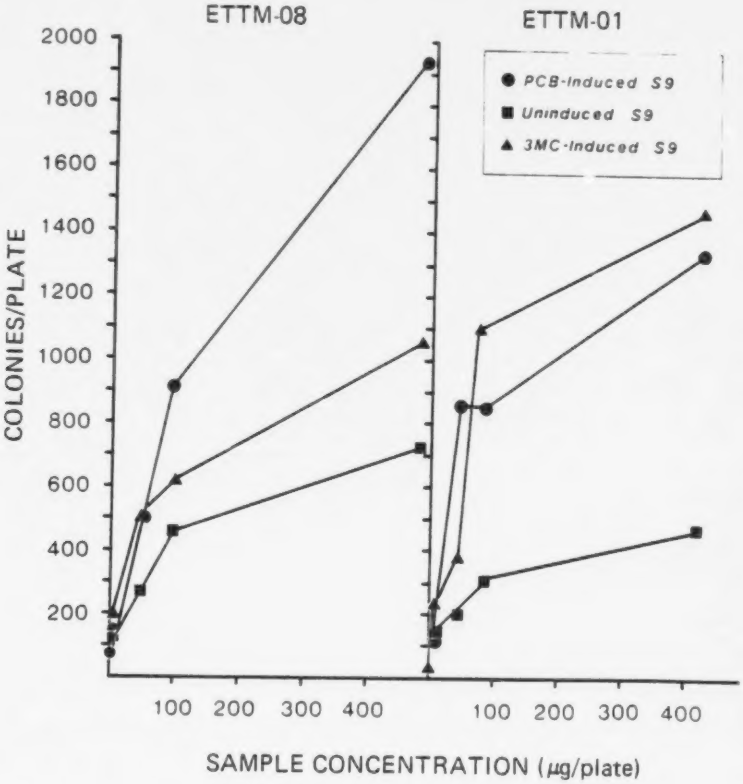

Figure 3. Effect of induction of S-9 (50 μl/plate) enzymes on sample mutagenicity for TA98.

mutagenic forms were generated by the extraction process, although this appears unlikely, as no direct-acting forms were produced.

While mutagenicity in this assay is not proof of a compound's carcinogenic potential, it does indicate an urgent need for further study. If the types of material represented by these samples are to be produced in large amounts or are found to be widespread in the environment, they may pose a significant health problem.

It is likely that production of synthetic fuels will increase, necessitating the use of in vitro assays to assess the health hazards of products, intermediate streams, and wastes of coal processes. Future work should include samples from alternative processes, feedstocks, and varying process conditions. Bioassay

Table 3. Liquefaction Product (ETTM-01)

A. Extract	I Percent Extracted[a]	II TA98 (colonies/mg)[b]	I x II Whole Sample Normalization (colonies/mg)
Hexane	29.09	7,640	2,222
Toluene	65.26	16,490	10,761
Methylene chloride	3.84	5,220	200
Acetonitrile	0.16	-	-
Residue	1.74	-	-
Sum of fractions	100		13,183 (74.5%)
Whole sample	100		17,690 (100%)

B.	No. of Data Points	Coefficient of Linear Regression
Whole	30	0.902
Hexane	34	0.975
Toluene	28	0.923
Methylene chloride	28	0.905

[a] Initial weight prior to extraction: ETTM-01A, 5.2948 g; ETTM-01B, 2.9320 g. Sum of organic extract weights: ETTM-01A, 5.4655 g (a gain of 3.22%); ETTM-01B, 3.2128 g (a gain of 9.60%).

[b] Calculated from linear portions of dose response curves: - = no dose response.

data from both complete and fractionated samples will be correlated with chemical characterizations to identify specific compounds or functionality effects. Such results will be compared with those from toxicology testing on larger-scale advanced coal processes. Assessing hazards early in the process-development cycle will facilitate development of technology to minimize health risks.

Table 4. Liquefaction Distillate Oils (ETTM-10)

A. Extract	I Percent Extracted[a]	II TA98 (colonies/mg)[b]	I x II Whole Sample Normalization (colonies/mg)
Hexane	97.47	9,500	9,260
Toluene	0.86	271,370	2,334
Methylene chloride	1.21	63,890	774
Acetonitrile	0.05	63,510	32
Residue	0.41	13,260	54
Sum of fractions	100		12,453 (134%)
Whole sample	100		9,280 (100%)

B.	No. of Data Points	Coefficient of Linear Regression
Whole	24	0.815
Hexane	31	0.937
Toluene	24	0.777
Methylene chloride	24	0.934
Acetonitrile	18	0.958
Residue	38	0.868

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[a] Initial weight prior to extraction: ETTM-10A, 5.0212 g; ETTM-10B, 4.7879 g. Sum of organic extract weights: ETTM-10A, 4.2488 g (a loss of 15.4%); ETTM-10B, 4.5672 g (a loss of 4.61%).

[b] Calculated from linear portions of dose response curves.

ACKNOWLEDGMENT

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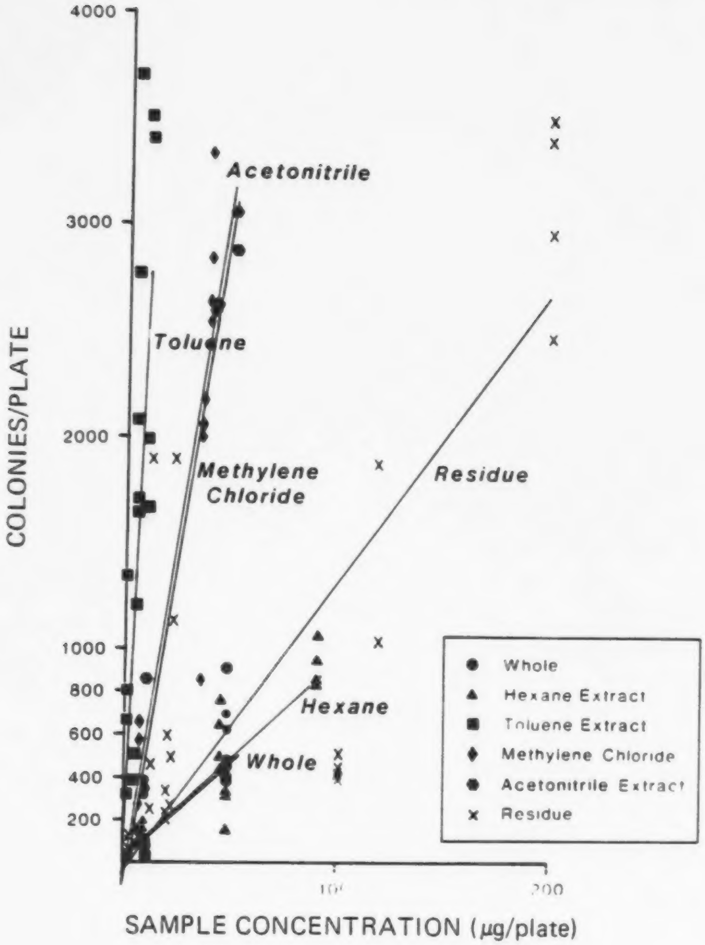

Figure 4. Mutagenicity of organic solvent extracts of ETTM-10 for strain TA98 with Aroclor-induced S-9 (50 μl/plate).

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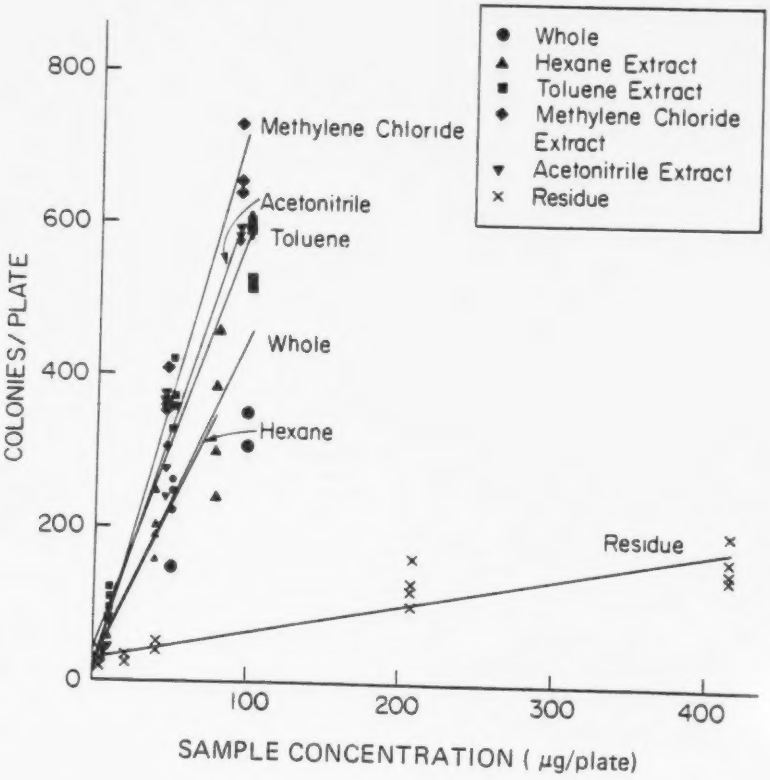

Figure 5. Mutagenicity of organic solvent extracts of ETTM-02B for strain TA98 with Aroclor-induced S-9 (50 μl/plate). Correlation coefficients (r) for the linear regressions are as follows: whole sample, 0.924; hexane extract, 0.940; toluene extract, 0.980; methylene chloride extract, 0.994; acetonitrile extract, 0.989; residue, 0.965.

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SESSION 6

HAZARD ASSESSMENT

PB82-233511

THE ROLE OF SHORT-TERM TESTS IN ASSESSING THE HUMAN HEALTH HAZARDS OF ENVIRONMENTAL CHEMICALS: AN OVERVIEW

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The problems to be addressed in this paper and in this session are twofold: first, to identify and confirm mutagenic and presumptive carcinogenic chemicals through short-term tests (based on the use of plant as well as animal materials): and second, to determine the relationship of demonstrated effects in short-term tests to actual hazards and risks to human health.

When we consider the complexity of environmental samples, it becomes clear that removing all chemical hazards is not feasible. Increased exposure to man-made carcinogens and mutagens in the environment must be presumed to entail additional human health risk. Therefore, our research objective and our regulatory objective must be to identify the potential hazards of such compounds and to minimize the risk to human health.

Even the task of identifying those chemicals that pose mutagenic or carcinogenic hazards is complex, because of the diversity of such agents and the multiplicity of their interactions with biological systems. Mutagens can induce heritable changes in the genetic material of either somatic or germinal cells of living organisms. Alterations can occur at the single gene and the chromosomal level. Many carcinogens act through mutational mechanisms, but some may act through other physiological changes that may alter, for example, the individual sensitivity or pattern of tumor expression. Eventually, we shall have to develop hazard assessment procedures that take into account the range of genotoxic and physiological properties of substances evaluated as carcinogens.

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Our understanding of the mechanistic basis of genetic toxic effects is undoubtedly best developed in the field of mutagenesis. Much of this information was obtained from the kinds of short-term systems that we are discussing. We know much less about the measurement of human genetic effects. Cytogenetic techniques have demonstrated that changes in structure and number of chromosomes are associated with a variety of human diseases. The cri-du-chat syndrome and one form of Down's syndrome are associated with structural alterations (a chromosomal deficiency and translocation, respectively). Kleinfelter's and Turner's syndromes are attributable to alterations in chromosome number. It is estimated that chromosomal abnormalities occur in the human population at a combined frequency of around 0.5% of all live births. Some 50% of all spontaneous abortions involve chromosomal defects. The relationship between heritable chromosomal alterations in man and exposure to environmental mutagens remains uncertain. But exposure of various experimental organisms to such agents definitely increases the frequency of chromosomal abnormalities.

Similarly, the induction of gene mutations by environmental chemicals is well known in experimental organisms. Such mutations give rise to new alleles that can have a variety of effects, depending on the mode of gene expression. In man, dominant alleles may be responsible for physical defects, such as dwarfism. It is estimated that dominant mutations may cause adverse effects in up to 0.5% of the human population. Perhaps of greater concern are the recessive mutations. Well over 1000 disease states display inheritance patterns characteristic of recessive mutations, including phenylketonuria (PKU), Tay-Sachs, and cystic fibrosis. The association of such genetic anomalies with chemically-induced mutation in man has not been established. However, since induced recessive mutations can remain unexpressed for many generations, exposures to environmental mutagens could be covertly increasing the genetic load of the human gene pool. Certainly human exposure to man-made mutagens has increased, and the genetic risk associated with such exposure must be evaluated.

Heritable genetic damage is not our only concern. The somatic mutation theory of carcinogenesis extends the concern to carcinogenesis as well. Fortunately, microbial mutagenesis tests, when coupled with mammalian metabolic activation, detect a major proportion of the chemicals that have been shown to be carcinogenic in animals. The overall qualitative correlation between the mutagenicity of chemicals in microbial systems and the carcinogenicity of the same chemicals in experimental animals is quite good. Limited data suggests that the quantitative correlation between mutagenic potency in mammalian cell systems and carcinogenic potency in animals may be somewhat better than in the case of the microbial systems, but fewer chemicals have been tested. Dr. de Serres discusses in his paper (1980) results of various mutagenicity and related tests conducted on a series of carcinogens and noncarcinogens.

To the extent that such correlations depend on more accurate measurement of metabolism, we have made several recent advances. Perfusion techniques and cell dissociation methods have made it possible to prepare metabolically active primary cells for co-cultivation with mutagenesis indicator systems or with cells that are subject to transformation *in vitro*. While these technical innovations may improve our ability to detect genetically toxic agents missed with microsomal activation methods, there is no substitute for the intact mammal, when mutagenic or carcinogenic potency must be quantified directly. In addition to pharmacokinetic considerations, species, strain, and sex differences must be considered, as well as organ specificity and differences in sensitivity of various cell types and cell stages. Ultimately, potency is a question of dose-response relationships and the probability of effects at environmental dose levels. Short-term tests that can be applied directly to man are highly desirable in this regard. Dr. Wyrobeck (1980) specifically addresses such tests using sperm. Similar procedures are being developed with laboratory animals. The ability to identify mutations in single cells of exposed animals will have significant advantages over presently available *in vitro* and *in vivo* mutagenesis bioassays.

Finally, I would like to discuss two major approaches to assessing genetic risk to humans resulting from exposure to chemical mutagens. One uses experimental data obtained directly from induced germinal mutations in intact animals, and the other uses data on chemical dose to the germ cells of animals together with mutagenesis results obtained with other-than-germinal cells in various short-term tests.

Tests that directly provide information on induced germinal mutations include the specific locus test, the X-Y chromosome loss test, and the heritable translocation test. These tests are complete, in that they measure genetic damage in germ cells which is expressed in the subsequent generation. This information on induced mutation frequency may be combined with information on levels of human exposure. To estimate human risk, the induced mutation frequency is extrapolated downward to the estimated level of human exposure. Theory suggests the use of a linear or no-threshold model for point- or gene-mutational effects. Chromosomal alterations, on the other hand, are thought to proceed by multi-hit mechanisms. Thus, linear extrapolation of translocation data is likely to overestimate risk at lower levels of exposure. Other models may be more appropriate when supported by sufficient data. As was mentioned earlier, gene mutations usually occur at lower doses than do chromosomal mutations;

therefore, gene mutation data would be expected to be more sensitive for human risk assessment.

The issue of sensitivity tends to favor the second approach to genetic risk assessment. Because short-term test systems, particularly the *in vitro* systems, are usually more powerful, it is possible to detect more-readily-induced mutants and to relate this number to the chemical estimation of mutagen-DNA interactions or binding to DNA. A similar determination can be made of mutagen-DNA interactions in the germinal cells of the intact animal that has been exposed via an appropriate route. With knowledge of mutagen-DNA interactions in the intact animal and in the short-term mutagenesis test, a relationship can be constructed between exposure in the one system and induced mutation frequency in the other. To the extent that binding of the test mutagen to DNA can be measured at anticipated human exposure levels, it may not be necessary to perform a high-to-low dose extrapolation. If extrapolation is required, it may be assumed that DNA binding is directly proportional to the exposure level, unless there is evidence to the contrary. It is extremely important to understand the relationship between exposure, DNA binding, and mutation in all of our short-term mutagenesis tests in order to make maximum use of resulting data for relative chemical potency evaluation and for hazard assessment. These same considerations of exposure, effective dose, and response apply as well to other genetically mediated effects.

As far as carcinogenesis is concerned, we are in a better position to address the issue of human risk assessment because we have epidemiological evidence of cancer in man. This evidence provides us with the necessary information on human exposure and response to validate our long-term whole animal models. On the strength of correlations with whole animal data, short-term test results provide evidence of the carinogenic potential of previously untested pure chemicals and complex mixtures. We have seen in this symposium that comparative studies of the relative biological activity of complex mixtures can be used to provide early information on the carcinogenic potential of such materials. However, at this stage in the development of short-term tests, their results are best considered suggestive rather than conclusive. Given the evidence from these tests and human exposure considerations, it is reasonable to require the performance of long-term whole animal tests for carcinogenesis and related effects to define more precisely the extent of human health risk. Dr. Albert, who is directly involved in the process of health risk assessment, elaborates on these points in his paper (1980).

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PB82-233529

THE INTERNATIONAL PROGRAM FOR THE EVALUATION OF SHORT-TERM TESTS FOR CARCINOGENICITY (IPESTTC)

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INTRODUCTION

The major impetus for developing the International Program for the Evaluation of Short-term Tests for Carcinogenicity (IPESTTC) was that our need for rapid identification and control of carcinogens is not satisfied by traditional rodent bioassays. Because of resource limitations, rodent studies cannot be carried out on a large enough scale to identify all carcinogenic chemicals in the environment within a reasonable period of time. This need places tremendous pressure on the scientific community to develop test systems for identifying chemical carcinogens in the environment at a lower cost and on a shorter time scale.

A major problem in selecting short-term tests for carcinogenicity has been that the mechanisms of action of chemical carcinogens have not been well understood. However, during the past decade, developments in genetic toxicology have allowed progress towards a unifying theory of the action of many carcinogens. This theory is based on the hypothesis that chemical carcinogens induce mutations in somatic cells, and that these mutations change the behavior of the cells so that cancer develops. It is a logical step from accepting the somatic mutation theory of cancer to using short-term mutation tests for detecting chemicals that potentially produce mutations and cancer. However, other theories of the induction of cancer exist, and many other highly accurate tests have been developed to identify carcinogens.

The availability of a large number of short-term tests has created a problem for both the scientist and administrator who must select the most appropriate and accurate test systems for

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carcinogenicity screening. During the past few years, various laboratories have tackled this problem using well-known validation studies. The purpose of these studies is to assess how effectively the short-term test can distinguish between carcinogens and noncarcinogens that have been classified as such by their activity in whole-animal systems. However, the problem remains of how to compare the performance of different test systems when the data describing the performance has been developed in different laboratories using tests with different protocols and different criteria.

IPESTTC was designed specifically to examine the abilities of various test systems to distinguish between known chemical carcinogens and known noncarcinogens. Although many of the test systems may also detect mutagenic events, this was not the primary purpose of this program. As the program developed, it became apparent that a secondary aim could be achieved, namely, providing a body of data on the mutagenicity of various chemicals. This body of data would not only describe the effects of the chemicals on various test systems, but would help to make clear what additional information was required to describe completely the biological activity of these chemicals.

HISTORICAL DEVELOPMENT OF IPESTTC

This program emerged from research on short-term tests supported by the United Kingdom Medical Research Council (MRC) under commission from the United Kingdom Health and Safety Executive (HSE). Early discussions on how best to carry out this study led to a proposal by scientists at the Imperial Chemicals Industries, Ltd. (ICI) to expand the study to include a larger number of chemicals, which would be tested blind as coded samples. ICI scientists took on the responsibility for selecting the chemicals and preparing them in a high state of purity and in large enough quantity that all investigators could work with samples taken from the same batch. As it has evolved, the program is a unique attempt to gather a large set of test results with which to objectively evaluate the ability of short-term tests to correctly distinguish between the carcinogens and noncarcinogens.

The initial selection of test systems included a wide variety of assays, but none involved inducing cancer in animals. In each case a correlation with carcinogenic activity in laboratory animals was sought. It was agreed that the most useful method for assessing the performance of short-term tests would be to compare the results for pairs of structually-related chemicals where one was a known carcinogen and the other a known noncarcinogen. The investigators would not know the identity of the chemicals tested; in other words, the test chemical would be evaluated as coded samples in a blind trial. Scientists of Imperial Chemicals Industries, Limited, agreed to supply the chemicals for this testing program and selected the 25 carcinogens and 17 noncarcinogens (including 14 paired compounds). All chemicals were synthesized and prepared for the study in as pure a state as possible and in large enough quantity that all investigators could use samples from the same batch. Since the 50-g quantities prepared exceeded the requirements of the original HSE/MRC program, the National Institute of Environmental Health Sciences (NIEHS) developed a plan to include a larger variety of test systems. This expansion made it possible to look for interlaboratory variation in test performance. In other cases, where the time required to test the set of 42 chemicals exceeded that allowed in the program plan, samples were divided among laboratories performing the same test, in an effort to make the final test data as complete as possible.

Many investigators financed their own testing. In other cases, the work was financed by the HSE/MRC science in the United Kingdom, NIEHS or the U.S. Environmental Protection Agency in North America, or various other mechanisms in other parts of the world such as Japan and the Soviet Union. By the time of the final meeting at St. Simons Island, GA, in October, 1979, 30 different assay systems were part of the program and data from over 50 laboratories were considered.

SELECTION OF TEST CHEMICALS

The three main criteria for selecting the test chemicals were 1) to have as large a range of chemical types and chemical classes as possible in a group of 42; 2) to ensure the highest possible purity and to ensure that all samples of each chemical under test would be taken from the same batch; and 3) to obtain a balance among different chemical classes, not including too many chemicals from any particular class. In addition, 11 of the 25 chemical carcinogens selected were included, because they were known to be difficult to detect as mutagens in assays for point mutation in either *Salmonella typhimurium* or *Escherichia coli*.

In the selection of noncarcinogens, an attempt was made to find structural analogs of the chosen carcinogens. When a test system gives a positive response to a carcinogen, one cannot determine whether the assay is responding to some chemical property other than the carcinogenicity inherent in the chemical structure. Therefore, the most meaningful assay systems for screening tests would be those that gave a positive response with a given carcinogen and a negative result with its noncarcinogenic structural analog. This is another reason for stressing purity of the test chemicals: to be sure that the noncarcinogens (for example) are not contaminated with trace levels of carcinogenic structural analogs. Such contamination would elicit a positive response for noncarcinogenic chemicals that should give negative results. All chemicals (except auramine) were more than 99% pure, and at least six different criteria of purity were evaluated.

The chemicals selected for testing are given in Table 1. This table lists the 25 carcinogens and the 11 of these 25 that are difficult to detect in bacteria. Also listed are the 17 noncarcinogens arranged in order of the strength of the data available in the literature for making this evaluation. Selection of the noncarcinogens proved exceptionally difficult and somewhat disappointing, since much attention has been given in the past to the development of criteria for carcinogenicity, but not noncarcinogenicity. In Table 2, the list of chemicals are reorganized to show the 14 pairs of carcinogens and noncarcinogenic analogs.

SELECTION OF ASSAY SYSTEMS

An effort was made to include representatives of all available types of short-term assays thought to be potentially useful carcinogenicity pre-screening tests. Several of the assays included in the study were part of the original HSE/MRC study in the United Kingdom. As the program grew, subsequent sponsors selected other assays to fill in gaps in the program. Many assays were included because of their sponsors' interest in participation and willingness to do so without financial support. The developmental status of the assays ranges from those considered to be the best-standardized and -validated (such as the *Salmonella*/microsome assay) to those in the earliest stages of development (such as the inductest and the diptheria toxin resistance system in human fibroblasts).

No effort was made to standardize protocols. Without knowledge of optimum protocols, standardization would only insure that each investigator using a given assay would make the same mistakes. As a result, the program allowed a comparison of results from different protocols. Among the investigators using established assays, general agreement on a protocol was reached for maximum comparability of the results (as with the sex-linked recessive lethal test in *Drosophila* and the micronucleus test in mice).

The assay systems may be divided into five groups as shown in Table 3. In group 1 are listed the two inductests used in the study; one assays the lysis of bacteria (*E. coli* and *B. subtilis*), the other, the expression of genes linked to prophage lambda. The other two tests are the degranulation test (which assays the dissociation of ribosomes or polysomes from rat liver endoplasmic

Table 1. Chemicals Selected for Testing in the International Program for the Evaluation of Short-term Tests for Carcinogenicity

Chemicals Classified as Carcinogens

4-Nitroquinoline-N-oxide
Benzo(a)pyrene
9,10-Dimethylanthracene[a]
2-Acetylaminofluorene
N-nitrosomorpholine
2-Naphthylamine
Hydrazine sulfate
Diethylstilbestrol[b]
Cyclophosphamide
3-Aminotriazole[b]
4-Dimethylaminoazobenzene (butter yellow)[b]
4,4'-Methylenebis-(2-chloroanilin) (MOCA)
Hexamethylphosphoramide (HMPA)[b]
Methylazoxymethanolacetate
Auramine (technical grade)[b]
Benzidine
β-Propiolactone
Chloroform[b]
Dimethylcarbamoyl chloride
Urethane[b]
DL-Ethionine[b]
Ethylenethiourea[b]
Safrole[b]
Epichlorohydrin
O-Toluidine hydrochloride[b]

Chemicals Classified as Noncarcinogens

Pyrene[a]
Anthracene[a]
4-Acetylaminofluorene[a]
1-Naphthylamine[d]
Azoxybenzene[e]
Sugar (sucrose)[e]
Dinitrosopentamethylene tetramine[e]
Isopropyl N(3-chlorophenyl) carbamate[c]
3-Methyl-4-nitroquinoline-N-oxide[e]
3,3',5,5'-Tetramethylbenzidine[e]
γ-Butyrolactone
1,1,1-Trichloroethane[d]
Dimethylformamide[d]
Diphenylnitrosamine[2]
Methionine[a]
Ascorbic acid[a]
4-Dimethylaminoazobenzene-4-sulfonic acid Na salt[d]

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[a] Data not convincing.
[b] Carcinogen difficult to detect in bacterial assays.
[c] Best evidence for noncarcinogenicity.
[d] Intermediate evidence.
[e] Poorest evidence for noncarcinogenicity.

Table 2. Pairs of Structural Analogs Among the Chemicals Tested

Carcinogen	Noncarcinogenic Analog
4-Nitroquinoline-N-oxide	3-Methyl-4-nitroquinoline-N-oxide
Benzidine	3,3',5,5'-Tetramethylbenzidine
4-Dimethylaminoazobenzene (butter yellow)	4-Dimethylaminoazobenzene-4-sulfonic acid Na salt
Benzo(a)pyrene	Pyrene
β-Propiolactone	γ-Butyrolactone
9,10-Dimethylanthracene	Anthracene
Chloroform	1,1,1-Trichloroethane
2-Acetylaminofluorene	4-Acetylaminofluorene
Dimethylcarbamoyl chloride	Dimethylformamide
2-Naphthylamine	1-Naphthylamine
N-Nitrosomorpholine	Dinitrosopentamethylene tetramine
Urethane	Isopropyl N(3-chlorophenyl)carbamate
Methylazoxymethanol acetate	Azoxybenzene
DL-Ethionine	Methionine

reticulum) and the nuclear enlargement assay (in which a positive result is indicated by an increase in the size of the nuclei in both HeLa cells and fibroblasts in culture).

Group II includes assays for the induction of point mutations in bacteria, including assays for forward and reverse mutation in both *Salmonella* and *E. coli*. The *Salmonella*/microsome reverse-mutation assay was conducted in 13 separate laboratories using a total of seven strains. Several procedures were used for metabolic activation, and the variations here included 1) source of S-9, 2) use of different chemicals as inducers, 3) variation in the amount of S-9, 4) plate incorporation versus pre-incubation, 5) the use of hepatocytes from rat liver, and 6) the addition of the comutagen norharman. Data were also obtained for a *Salmonella* assay that measured forward mutations to azaguanine resistance. In *E. coli*, two systems were included: one in which both forward and reverse mutation at four loci are screened simultaneously and another in which tryptophan reversion is measured in different strains of *E. coli* strain WP2.

The tests in group III measure various types of genetic damage in yeast, including forward mutation in *Saccharomyces pombe* and the following endpoints in *S. cerevisiae*: reverse mutation, mitotic crossing over in five different strains, induction of aneuploidy in strain D6, and differential survival of wild type and a multiply repair-deficient strain.

Table 3. Assay Systems Used in the International Program for the Evaluation of Short-term Tests for Carcinogenicity

I. Prokaryotic Repair, Phage Induction, Nongenetic Assays	
Inductests: *Bacillus subtilis* rec assay *Escherichia coli*	*Escherichia coli* pol assay Nuclear enlargement Degranulation test
II. Prokaryotic Mutation Assays	
Salmonella/microsome fluctuation assay *Salmonella* 8-AZA resistance	*Escherichia coli* 343
III. Yeast Assays	
Forward mutation--*S. pombe* Reverse mutation--XV185-14C Mitotic recombination - PG-154, PG-155, D4, D7, JD1	Aneuploidy - D6 Repair assay - RAD, URA
IV. Mammalian *In Vitro* Assays	
Unscheduled DNA synthesis (WI-38, HeLa) Sister-chromatid exchange (CHO) Chromosome aberrations (CHO, RL_1) BHK21--transformation	CHO-HGPRT, APRT, TK, OUS V79-HGPRT Human fibroblasts--diphtheria toxin resistance
V. *In Vivo* Assays	
Sex-linked recessive lethal--*Drosophila* Sister-chromatid exchange--mouse	Micronucleus--mouse Sperm morphology--mouse

The assays in group IV are mammalian *in vitro* systems, including assays for gene mutation in Chinese hamster, V79, mouse lymphoma, and human fibroblast cells. Chromosome effects *in vitro* include sister-chromatid exchange, chromosome aberrations, and unscheduled DNA synthesis. The BHK21 cell transformation assay based on growth in soft agar is also in this group of assays.

The Group V assays are whole-animal systems, including sex-linked recessive lethals in *Drosophila*, the micronucleus test in mice, and sister-chromatid exchange in liver cells and bone marrow. The only plant system included in the program is the induction of gene mutations resulting in color changes in *Tradescantia* stamen hairs.

EVALUATION OF THE DATA BASE

The data base that has come out of this program is broad and complex. Eventually, it can be used not only to compare the performances of the individual assay systems, but also to evaluate the effects of the chemicals both qualitatively and quantitatively. One of the difficulties in comparing the performances of the various assay systems is that due to resource limitations, not all 42 chemicals were tested in each assay. Since the samples were coded, the chemicals tested should be a random sample of the 42. However, the chemicals were usually tested in order of their receipt, so that the gaps in the data base involve the same group or groups of chemicals. Resource limitations also precluded interlaboratory comparisons of each assay system.

Each assay system's performance has been evaluated (Table 4) by its ability to correctly classify as positive the carcinogenic chemicals (sensitivity) and as negative the noncarcinogenic chemicals (specificity) and by its overall ability to correctly classify both carcinogens and noncarcinogens (accuracy). It should

Table 4. Terms Used in Describing Assay Performances

$$\text{Sensitivity (true positive fraction)} = \frac{\#\text{ positive results with carcinogens}}{\#\text{ carcinogens tested}}$$

$$\text{Specificity (true negative fraction)} = \frac{\#\text{ negative results with noncarcinogens}}{\#\text{ noncarcinogens tested}}$$

$$\text{Accuracy} = \frac{\#\text{ correct results}}{\#\text{ chemicals tested}}$$

be remembered that two assay systems can have the same sensitivity, specificity, and accuracy, and yet differ in the sets of chemicals that they correctly classify.

In the present study, 11 of the 25 carcinogens were selected because they are difficult to detect as mutagens in the bacterial assays for point mutation. Because of this, the sensitivity, specificity, and accuracy figures will differ markedly from previous studies. The data from the present program are being selectively analyzed so that these comparisons can be made for selected portions of the data base, with groups of chemicals omitted or with their classifications changed.

The calculations for the 42 chemicals show that no single assay system is sufficiently sensitive, specific, and accurate to be used in isolation. The data clearly indicate the need for a battery of tests.

EVALUATION OF TEST DATA ON THE 42 CHEMICALS

The main problem with this evaluation was that six of the noncarcinogens may not have been correctly classified. The following six chemicals gave a high frequency of positive results over a wide range of assay systems: 3-methyl-4-nitroquinoline-N-oxide, 4-acetylaminofluorene, 1-naphthylamine, azoxybenzene, diphenylnitrosamine, and 4-dimethylaminoazobenzene sulfonic acid (methyl orange). Those results indicate that they are probably carcinogens and are thus misclassified for the purpose of the present program. An advantage of computerization of the IPESTTC data is that the classification of these six chemicals can be changed and new estimates of sensitivity, specificity, and accuracy obtained.

PROGRAM COORDINATION AND COMPLETION

IPESTTC was managed by a coordinating committee consisting of F.J. de Serres, Chairman, J. Ashby, P. Brookes, B. Bridges, I. Purchase, M. Shelby, and T. Sugimura. This group was responsible for the initial selection of assay systems and investigators, distribution of test samples, collection of data, and organization of the various workshops held during the course of the study. In addition, this group was responsible for the follow-up work required to complete the present data base, as well as its final analysis.

An interim report of this program will be published as a book (de Serres and Ashby, in press) containing reports from all of the investigators. It will also contain summary reports from the various test-system and test-chemical work groups that met to evaluate the data base after decoding of the test chemicals. The data, along with more detailed discussion, will be published early in 1981.

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SPERM ASSAYS IN MAN AND OTHER MAMMALS AS INDICATORS OF CHEMICALLY INDUCED TESTICULAR DYSFUNCTION

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INTRODUCTION

Concern about human exposure to chemical agents has led to the development of numerous bioassays to detect mutagens and carcinogens rapidly and inexpensively. Recent attempts to compare and evaluate the efficacy of these bioassays have shown clearly that no single assay is sufficient (Coordinating Committee, 1978). A number of sperm assays have been developed and evaluated (Wyrobek, in press). Although the mutagenic basis of chemically induced sperm anomalies is generally not well understood, these assays play an important role in mutagenesis and carcinogenesis testing. First, sperm assays can measure chemical damage to the germ cells occurring during spermatogenesis or transit through the efferent ducts. Agents that are found to be mutagenic or carcinogenic in other bioassays can be tested directly for their spermatotoxic effects. This possibility is of major importance because the activity of an agent in bacteria or mammalian somatic cells is often a poor predictor of its activity in the testes after exposure *in vivo* (Coordinating Committee, 1978). Second, since animal sperm assays are as inexpensive as other short-term tests, many agents can be tested. Third, sperm assays have not only been developed and applied to mice, other rodents, and a variety of domestic animals, but they are also applicable to men exposed to chemicals (Wyrobek and Glendhill, in press).

This paper describes the application and methodology of sperm assays in men and laboratory animals and discusses the predictive value of induced sperm changes, correlations of results to carcinogenicity and mutagenicity, and the relative strengths and weaknesses of sperm assays (for more detailed accounts with specific agents, see Topham, 1980b: Wyrobek and Bruce, 1975: Wyrobek et al., in press b, c).

METHODOLOGY

Human Sperm Assays

Human semen assays have a long history in the diagnosis of infertility. Thus, it is not surprising that early attempts to assess altered testicular function in men exposed to chemicals have involved measuring changes in the sperm parameters commonly used in fertility diagnosis, such as sperm density (counts), motility, and morphology.

Of these parameters, morphology is the most constant in an unexposed man and is statistically highly sensitive to small changes (Wyrobek et al., in press c). In the past, this assay has not been widely used, because the scoring criteria were generally difficult to standardize, and interlaboratory discrepancies were unavoidable. We have improved the human morphology assay by describing 10 classes of spermhead shapes and categorizing at least 500 sperm per individual into one of these classes. Through the intermittent use of coded standard slides, we have shown constancy in scoring the same set of standard slides for up to three years and have demonstrated the objective nature of the scoring criteria (Wyrobek, in press). We have adapted this method to men occupationally exposed to carbaryl (Wyrobek et al., in press e), anesthetic gases (Wyrobek et al., in press a), dibromochloropropane (DBCP), and mercury (Wyrobek, et al., in preparation). Men exposed to carbaryl and DBCP showed marked sperm changes when compared with unexposed men. Men exposed to cancer-chemotherapeutic agents showed drug-related decreases in sperm counts and increases in sperm shape abnormalities (Wyrobek et al., 1980).

Among the more recently developed human sperm assays is the YFF test, which is based on the unique fluorescence of the human Y chromosome when stained with quinacrine (Kapp, 1979). Men exposed to adriamycin and DBCP, for example, showed exposure-related increases in the proportion of sperm having two fluorescent spots, which are presumably due to the presence of two Y chromosomes in one sperm because of errors in meiotic disjunction (Kapp, 1979).

A recent survey of the literature (Wyrobek et al., in press c) showed that human sperm assays have been more widely used than was generally suspected: more than 80 papers were found on the use of semen assays in assessing testicular function. About 75% of the exposures involved experimental or therapeutic drugs; about 15% were occupational exposures; and about 10% involved personal drug use. The studies cover 37 single agents, 10 complex mixtures, and

12 sets of multiple agents. Tables 1 and 2 list the single agents and complex mixtures categorized by those agents that 1) were found to induce significant changes in the sperm parameters of exposed men, 2) gave suggestive but inconclusive evidence of change, and 3) had no effect. Of the 37 single agents, 21 were positive, 9 suggestive, and 6 negative. Almost all of the agents showing some effect had a reduction in semen quality (e.g., reduction in sperm counts, decrease in sperm motility, decrease in the proportion of sperm with normal shapes, or increased frequencies of YFF sperm). However, five agents (chlomiphene citrate, coenzyme Q-7, fluoxymestrone, kallikrein, and methadone) were found to increase sperm counts and/or motility in some of the infertile patients studied.

Table 1. Single Agents Studied with Human Sperm Assays[a]

Positive Effects	Suggestive but Inconclusive Effects	No Effects Observed
Aspartic acid	Centchroman	CIBA-32644 Ba
Chlorambucil	Colchicine	Lysine
Chlomiphene citrate	Methadone	Methyl testosterone
Cyclophosphamide	Methotrexate	Ornithine
Cyproterone acetate	Metronidazol	Testosterone
Doxorubin hydrochloride	Nitrofurantoin	WIN 59491
Enovid	Trimeprimine	
Gossypol		
6 Medroxyprogesterone		
Metandienone		
Nilevar		
Norlutin		
Prednisone		
Progesterone		
Salicylazosulfapyride		
Testosterone enanthate		
Testosterone propionate		
WIN 13099		
WIN 17416		
WIN 18446		

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[a] For data on specific agents and their chemical names, see Wyrobek et al. (in press c). Table entries are based on studies of sperm counts, motility, morphology, and YFF. The assignment of individual agents to columns is based on the data provided in the papers reviewed by the U.S. Environmental Protection Agency (EPA) Gene-Tox panel (see Waters, 1979) and may change as more data becomes available.

Table 2. Complex Mixtures Studied with Human Sperm Assays[a]

Positive Effects	Suggestive but Inconclusive Effects	No Effects Observed
Alchoholic beverages	Carbaryl[b]	Epichlorohydrin[b]
Carbon disulfide[b]	Diethylstilbestrol	Glycerine workers[b]
Dibromochloropropane[b]	Tobacco smoke	Polybrominated biphenyls[b]
Lead[b]		
Marijuana		

[a] For data on specific agents and their chemical names, see Wyrobek et al. (in press c). Table entries are based on studies of sperm counts, motility, and YFF. The assignment of individual mixtures to columns is based on the data provided in the papers reviewed by the EPA Gene-Tox panel (Waters, 1979) and may change as more data becomes available.

[b] Occupational exposures.

Eleven complex mixtures were studied using the sperm assays. As shown in Table 2, five showed positive effects, three showed suggestive but inconclusive effects, and three showed no effect. Most of these studies involved occupational exposures in which single active agents were implicated (e.g., DBCP or lead).

Sperm assays have also been used in men exposed to at least 12 sets of two or more agents in consort (Wyrobek et al., in press c). Ten combinations caused reductions in semen quality (e.g., cyclophosphamide plus prednisone, Danazol plus testosterone enanthate, and MVPP cancer therapy).

Of the 60 different human exposures evaluated, including all single agents, multiple agents, and complex mixtures, 97% used sperm counts as one of the parameters measured, of which 25% used counts as the only parameter measured. Furthermore, 58% of all the exposures evaluated studied motility, 42% studied morphology, and only 7% used YFF.

Because of our poor understanding of the genetic mechanisms underlying various induced sperm anomalies, the only information that can be gained from these assays at present is whether human spermatogenesis is affected by exposure to a chemical agent or mixture of agents. These data, together with results of other short-term assays for mutagenicity, may indicate which of these agents are potential germ cell mutagens. Clearly, more research is needed to develop sensitive sperm assays with defined mutational end points, so that risks of heritable damage may be assessed directly using sperm.

Animal Sperm Assays

Sperm assays have also been used to assess chemically induced changes in testicular function in a variety of animal species. At least 13 agents have been studied in rabbits, 14 in rats, 2 each in sheep, cattle, dogs, hamsters, and monkeys, and 1 in pigs (for review, see Wyrobek et al., in press b). Most of the studies (covering approximately 159 agents) used the mouse sperm morphology assay (Wyrobek and Bruce, 1975). Approximately 40 agents produced dose-dependent increases in sperm shape abnormalities, 105 were negative, and 5 showed marginal responses. However, of the negatives, only about 50 were known to have been tested to lethal doses, and the remainder should be retested at higher doses. Positive results came from a wide variety of chemical classes, including antimetabolites, alkylating agents, spindle poisons, polyaromatic hydrocarbons, aromatic amines, estrogens, and others. Negative responses will occur with any compound that rapidly kills the animal or whose active form does not reach the testes either because of the route of exposure or the metabolism required to activate the agent. Because only seven of the chemical agents reviewed were tested in two or more species, meaningful interspecies comparisons of results of sperm assays are not yet possible.

DISCUSSION

The Genetic Implications of Chemically Induced Sperm Anomalies

Evidence from human studies. Although it is generally agreed that major reductions in sperm counts and motility are linked to reduced fertility, it remains unclear which sperm parameter(s) indicates embryonic failure or heritable genetic abnormalities. Human data on this question is very limited. In one study, fathers of 201 spontaneous abortions showed significantly higher sperm abnormalities and lower sperm counts than 116 fathers of normal pregnancies (Furuhjelm et al., 1962), suggesting a link between poor semen quality and frequency of spontaneous abortions. Although several studies support this observation (Czeizel et al., 1976; Joel, 1966; Takala, 1957), some studies found no such correlation (Kneer, 1957). Clearly, more human studies are needed to compare exposure of the male parent, induced sperm defects, and reproductive outcome.

Evidence from animal studies. Most of the studies on genetic validation of induced sperm abnormalities have been conducted with mice. Three lines of evidence link induction of abnormal sperm and heritable genetic abnormalities. First, it is clear that sperm shaping and the production of abnormal sperm is polygenically controlled by autosomal as well as sex-linked genes (Beatty, 1972; Brozek, 1970; Hugenholtz and Bruce, 1979; Krzanowska, 1972; Topham, 1980a; Wyrobek and Bruce, 1978; Wyrobek, 1979). Second, in at least three independent studies with numerous mutagens and nonmutagens, germ cell mutagens generally induced sperm abnormalities, while nonmutagens generally had no effect (Bruce and Heddle, 1979; Topham, 1980b; Wyrobek and Bruce, 1978). Third, in several studies using agents that induce sperm abnormalities, sperm abnormalities were transmitted to the male offspring of the exposed mice (Hugenholtz and Bruce, 1979; Sotomayor, 1979; Staub and Matter, 1976; Topham, 1980a; Wyrobek and Bruce, 1978).

A brief survey of the literature indicates that many of the compounds that are active in the mouse sperm morphology test are also active in the heritable specific locus, F_1 sperm morphology, heritable translocation, and/or dominant lethal tests in mice (for review, see Wyrobek et al., in press b). Therefore, the mouse sperm morphology test may be a useful screening test for compounds that constitute a potential genetic hazard for mammals. Spindle poisons that may cause nondisjunction in germ cells can also be identified. Further murine studies are needed to understand the quantitative relationships among dosage regime, appearance of abnormal sperm shapes in the semen, time between exposure and conception, fertility of the exposed male, frequency of genetically abnormal offspring, and fertility of the abnormal offspring.

Correlations of Sperm Abnormality Results with Results of Short-term Assays for Carcinogenesis

Several attempts have been made to compare results of the murine sperm morphology assay with other short-term tests for carcinogenesis. As part of the International Program for the Evaluation of Short-term Tests for Carcinogenesis, six pairs of carcinogens and noncarcinogens and five unpaired carcinogens were surveyed as unknowns, using the mouse sperm abnormality assay (Wyrobek et al., in press d). No false positive responses were found, suggesting that the sperm assay has a high specificity for carcinogens. However, several false negatives were obtained, indicating that not all carcinogens induce sperm abnormalities in mice. This data may be very important in assessing which carcinogens may also be active in the testes. The detailed comparison of the sperm abnormality assay and the other assays surveyed in this program is still in progress.

A comparison of the potency (dose required to double background frequencies) of some 30 agents studied in both the Salmonella/microsome and sperm abnormality assays showed no apparent correlation, suggesting that the assays are measuring different biological phenomena (unpublished results). In a different study of 61 agents (Bruce and Heddle, 1979) with the mouse sperm morphology and Salmonella/microsome assays, each assay was found to correctly identify approximately 60% of the carcinogens and noncarcinognens tested. The assays together identified nearly 90% of the agents, suggesting that the two assays measure different end points. These authors recommended that the number of false negatives, which is relatively high with each of the two assays, may be reduced by using a battery of both assays for the identification of potential carcinogens.

CONCLUSIONS

Advantages

The major advantages of sperm assays are that the cells examined are readily available in both animals and man, and that sperm carry the paternal genome in the form that will be ultimately involved in fertilization. Other advantages are the following:

1) Sperm are examined after exposure of a whole mammal. This helps ensure that artifacts (false positives and false negatives) due to problems of tissue penetration, metabolism, pharmacokinetics, and dosage encountered in non-gonadal, cultured-cell, or nonmammalian systems are minimized.

2) The changes in sperm parameters probably arise from interference by the test substance with the differentiation of the sperm cell. Thus, these changes are intrinsically relevant to safety evaluation and assessment of potential effects of the agent on male fertility.

3) The laboratory methods are generally rapid, inexpensive, and quantitative.

4) Sperm assays have major advantages over other approaches for assessing induced changes in testicular function. Testicular biopsies are impractical, traumatic, invasive, and may themselves affect testicular function. Epidemiological surveys of reproductive function using questionnaires exclusively require large sample sizes and are generally expensive. Analyses of blood levels of gonadotrophins are expensive and generally insensitive to

small changes in testicular function. Compared with these methods, sperm assays are noninvasive, inexpensive, require small sample sizes for effective analyses, and are sensitive to small changes.

Disadvantages

The major disadvantages of sperm assays are the following:

1) Heritability of the induced damage is not yet clearly demonstrated.

2) Limited sperm sampling times and dosage regimens may reduce the sensitivity of the assay (e.g., agents that only exert transient effects may be missed by using single sampling times).

3) Other factors such as ischemia, infection, and starvation may produce spurious false positive responses.

Applications

The availability of animal and human sperm assays suggests several applications in the assessment of chemically induced spermatotoxicity (antifertility effects) and heritable genetic abnormalities. Animal sperm assays (such as the mouse morphology assay) may be used to screen large numbers of agents to establish a ranking that sets priorities for sperm studies in exposed men. This approach would minimize the use of human studies that generally have complex requirements for epidemiological and statistical input and often require lengthy interactions with union officials, industry representatives, employees, physicians, and patient-donors. Furthermore, animal sperm studies also may be useful in evaluating the relative effects of the components of a complex mixture that is suspected of affecting human sperm (such as an occupational exposure).

Since little is known of the quantitative relationships between induced sperm abnormalities and heritable genetic damage, indirect methods are needed to assess the genetic risk to offspring of men who show induced sperm anomalies. By combining data from short-term mutagen bioassays (e.g., Salmonella/microsome assay, mammalian somatic cell mutation assays), which may demonstrate mutagenic potential, with data from animal and human sperm assays, which may demonstrate activity in the testes, we may be able to evaluate whether a mutagen (or carcinogen) is active in the testes. For select agents, a more objective assessment of germ cell mutagenicity may be required. This could be done using various murine F_1 generation bioassays (e.g., heritable chromosomal translocation, dominant skeletal mutations, and heritable sperm abnormalities) to quantify the relationships between heritable consequences and chemically induced sperm anomalies. The combined use of data from animal and human sperm assays, short-term *in vitro* bioassays, and murine F_1 generation mutational bioassays may provide a feasible approach to genetic risk assessment in men exposed to agents that cause sperm anomalies.

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ASSESSING CARCINOGENIC RISK RESULTING FROM COMPLEX MIXTURES

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The evaluation of carcinogenic risks from complex mixtures, in contrast to pure substances, adds a dimension of uncertainty to a situation already frought with uncertainties and complexities.s. The uncertainties of evaluating complex mixtures are likely to be lost in the overall uncertainties of the risk assessment process. There is considerable controversy about the risk assessment of carcinogenic substances these days. Some liken the assessment of carcinogenic risks to the theater, in that a willing suspension of disbelief is required. To others, like myself, the assessment of carcinogenic risks reflects Mark Twain's definition of work: "It is something which a body is obliged to do." I believe that the risk assessment of carcinogens is something that one is obliged to do in a regulatory setting in order to make regulatory judgements in as rational a manner as possible. The responsibility of those doing the risk assessment is to make the most sensible use of current science, paying proper regard to cautioning those who are making the decisions about the uncertainties in the process.

One of the problems with the assessment of carcinogenic risks either of pure substances or complex mixtures is that it is a relatively new field. Before 1970, the only well-established risk assessment area involved ionizing radiation. It is worth noting that the standards for permissible exposure to ionizing radiation were set not on the basis of risk assessment but on the traditional basis of applying safety factors to observed levels of carcinogenic responses, particularly to cancer induction in the bone, lung, and bone marrow. It was in the field of ionizing radiation that the dominant concept of risk assessment emerged, namely, the linear non-threshold extrapolation model. This model was based on the correlation between carcinogenesis and mutagenesis and on the recognition that the linearity of dose response is applicable to mutagenesis and consistent with linearity in the dose response for leukemia, particularly among Japanese atom-bomb survivors.

The linear non-threshold concept has proven to be a powerful tool for predicting small excess risks of cancer at levels that can't be confirmed or denied by direct observation either in animals or in epidemiologic follow-up studies of exposed populations. Both animal studies and the epidemiologic follow-up methods are too insensitive to detect levels of risk below a few percent, levels which are far too high to be tolerated willingly. Consequently, the linear non-threshold concept of dose response can be considered a two-edged sword. It provides the impetus to regulate, because we are essentially accepting the position that any exposure, however small, will produce an excess of cancer, but it leaves us in a quandary as how to regulate, because it puts us in a position of trying to figure out which excess risks are tolerable under given circumstances. The non-threshold concept was first incorporated into the regulation of carcinogens in the Delaney amendment, which bans food additives that show evidence of carcinogenic action. The Delaney amendment reflects the scientific concept that there is no safe level of carcinogen exposure and the political judgement that no excess risk of cancer from food additives is tolerable.

In the 1970's the regulatory movement was accelerated with the formation of the U.S. Environmental Protection Agency (EPA), the Occupational Safety and Health Administration (OSHA), and the Consumer Protection and Safety Commission (CPSC). Many laws dealing with the regulation of carcinogens were formulated using different approaches to the control of carcinogens: the Clean Air Act calls for protecting everyone with a margin of safety--clearly impossible under a non-threshold concept; the Federal Insecticide, Fungicide, and Rodenticide Act calls for weighing risks and benefits; the Toxic Substances Control Act refers to making regulatory judgments based on reasonable risk; the Occupational Safety and Health Act calls for using "best available technology" combined with economic considerations. A number of other laws call for the use of "best available technology."

These different regulatory approaches use risk assessment in different ways and to different degrees. Some of them simply call for the characterization of an agent as a carcinogen with the application of "best available technology," thereby applying considerable economic and technical pressure. Others call for weighing risks and benefits, whereby an agent is not only characterized as a carcinogen, but its public health impact is estimated as a basis for evaluating the benefits and costs of regulation.

In 1976, EPA developed its own guidelines for the risk assessment of carcinogens in response to a great deal of criticism of its approach to regulating pesticides. The EPA guidelines for risk assessment are based on the evidence approach. Risk assessment was viewed as an exercise trying to answer two questions: First, is the agent likely to be a human carcinogen? Second, if the agent is a carcinogen, how much cancer is likely to be produced? The former question requires a qualitative judgment, the latter a quantitative one.

The qualitative judgement is based on the sum of evidence concerning the quality, scope, and kinds of tumorigenic responses. The judgements can range from the characterization of an agent with strong evidence, based on good epidemiologic data backed by animal data, to the opposite end of the spectrum where only a single test, a single strain, a single sex, or a single species may have a marginal response. The guidelines regard short-term tests as being in a suggestive category when they stand alone, yet recognize their great value in providing support for animal bioassays or epidemiologic evidence of carcinogenicity. It is difficult to know when a scientific approach achieves sufficient consensus to provide the basis for regulatory action. So far, short-term bioassays haven't achieved this stature. I am not sure when they will. A tremendous amount of work is certainly aimed in that direction. It may very well be that within a forseeable time, short-term bioassays will have sufficient stature to provide a very strong impetus toward regulation on their own merits.

The quantitative assessment looks at how much cancer is likely to be produced, and assumes some background knowledge about exposure. From our experience in the Carcinogen Assessment Group, I think that exposure assessment is one of the weakest areas in EPA. I am sure that this situation also exists in other agencies. A quantitative assessment is based on an estimate of the exposure as well as the use of models for extrapolation from high doses to low doses; in many cases it also involves extrapolation from animals to humans. The U.S. Environmental Protection Agency started off in its guidelines calling for the use of more than one extrapolation model. Over the last four years, the assessments have focussed on the linear non-threshold extrapolation model. While no one model has a resoundingly solid scientific foundation, the linear non-threshold model has more scientific plausibility than other models and also tends to provide conservative estimates of risk. This model has been used almost exclusively in EPA's Carcinogen Assessment Group over the past four years. Given the uncertainties in the quantitative assessment of risk, the linear non-threshold model can be regarded as providing a plausible upper limit of estimated risk.

This assessment approach has been used in EPA for the last four years on about 100 agents. More recently, an Interagency Regulatory Liaison Group looked at the assessment of carcinogenic risks and formulated a position document. The agencies represented were EPA, OSHA, CPSC, and the Food and Drug Administration. The position taken in this interagency document can be read to be essentially consistent with the approach that I already mentioned. Realistically, however, it reflects considerable resistance to quantitative risk assessment. The document, to a considerable extent, gives only qualified support to quantitative risk assessment and can be cited as support by agencies that wish to do or not do quantitative assessment, depending on their point of view. More recently the Federal Regulatory Council published a cancer policy: its approach to the assessment of carcinogens supports the approach that has been taken by the EPA and IRLG, but with more qualification of the quantitative aspects of risk assessment than has been given by EPA.

The assessment of complex mixtures is no different, at least in principle, to the pattern that I have described already. There is certainly nothing unusual about complex mixtures of carcinogens. After all, the first demonstration of chemical carcinogenesis in animals was in the 1920's by the Japanese Yamagiwa and Ichikawa. They painted coal tar on rabbits' ears and first displayed the action of chemical carcinogens. One hundred and fifty years earlier the first epidemiologic observations on cancer induced by environmental chemicals dealt with scrotal cancer caused by soot in chimney sweeps. Soot is a complex mixture. From the beginning, the field of chemical carcinogenesis has been firmly embedded in the problem of complex mixtures.

Probably the most important exercise that has been undertaken by the EPA Carcinogen Assessment Group involving complex mixtures has concerned diesel exhaust particulates. The approach here has been to peg the evaluation of diesel particulates to some fairly solidly established epidemiologic dose-response data for agents that are chemically similar to diesel exhaust, namely, lung cancer among coke-oven workers, cigarette-smoke-induced lung cancer, and lung cancer in workers who have used coal tar and asphalt roofing materials. The approach has been to develop a cross comparison of the potency of diesel particulate exhaust and the other three materials on the basis of short-term *in vitro* assays, mouse skin tumorigenesis studies, and intratracheal intubation studies in hamsters. If we can bracket the carcinogenic potency of diesel exhaust with respect to the materials that have been observed to produce lung cancer in humans, we can use the human epidemiology data as surrogates for diesel particulates data in quantitative risk assessment. I think that this approach covers about as much as can be done in terms of dealing with complex mixtures of this sort. The same principle could be applied to the comparison of complex mixtures with pure materials using animal studies; certainly the use of short-term assays, both in vitro and in vivo, is particularly important when complex mixtures have a variable composition.

The diesel story is certainly not yet finished. The research program is still in the stage of producing data. In addition to the efforts involving cross comparisons of different materials with diesel particulates for carcinogenic potencies, there are also studies dealing with exposure to the diesel exhaust per se. One should wait until much of the experimental data is in hand before making any firm quantitative risk assessments.

The risk assessment process is receiving its major challenge in the quantitative area, although the publicity over the recent saccharin epidemiology studies is probably going to provide ammunition to those who are opposed to the use of assessment even in the qualitative area, namely, the applicability of rodent bioassays to human cancers. I must express my dismay at the way in which the publicity about the saccharin studies failed to highlight the fact that the duration of exposure to saccharin is completely inadequate to permit a proper characterization of its carcinogenicity in humans. It is perfectly clear that even if one were dealing with an agent that produced cancer, if the effects on human populations were evaluated before the end of the latent period, no effects would be found.

But the most serious challenges to risk assessment come in the quantitative area. I have mentioned the kind of objections that have been made by some agencies within the IRLG; also, the Federal Regulatory Council tended to downplay quantitative risk assessment on the basis of its uncertainty. Industry doesn't particularly like the EPA brand of quantitative risk assessment because of the use of the conservative linear non-threshold dose-response model. I think the position of industry is that if one is going to do quantitative risk assessment, which they think is not a bad idea, it would be better to use models that are less conservative, namely that show a curvilinear dose response and yield much lower risk estimates. The Occupational Safety and Health Administration is against quantitative risk assessment, because it feels that the law doesn't require it and it would only interfere with its regulatory program. There will be a decision made on this question by the Supreme Court in the case of benzene. The National Academy of Science, in a yet-to-be-released document on how to regulate pesticides, also takes a crack at quantitative risk assessment. This particular committee would go so far as to agree that one could extrapolate from animals to humans, as a basis for characterizing carcinogens in terms of relative potency, but they balk at the use of extrapolation models to predict responses at low levels of exposure. It has been pointed out, to no effect, that several other committees in the National Academy of Science have used the linear non-threshold extrapolation model for estimating risks. Probably the most vocal group to express opposition at the present time to quantitative risk assessment is the environmentalists. In a joint comment by the Environmental Defense Fund and the Natural Resources Defense Council to EPA on its newly proposed Air Cancer Policy, they say that "EPA must abandon its arbitrary and unlawful reliance on quantitative risk assessment methods. Quantitative risk estimates can be wrong by a factor of five million times or more and thus are too unreliable and imprecise to play a rational role in determining the levels of control applied to a hazardous pollutant. Such estimates may have a role in the grossest form of priority setting, but that will be valid only if the agency much more explicitly recognizes the uncertainties of the estimates and commits itself to not using them in any way in subsequent standard setting."

That quote is quite a forceful expression of opinion. The notion that quantitative risk estimates can be wrong by a factor of five million stems from the National Academy of Sciences Saccharin Report, which listed the risk estimates from saccharin using a variety of extrapolation models. One can pick the extrapolation model that yields the kind of result that one wants. It is quite easy to pick a set of models that give estimates that vary by many orders of magnitude. Although I do believe that it is fair to say that the use of the linear non-threshold extrapolation model may somewhat overestimate risk, it is not likely to produce much of an underestimate of risk. Certainly, for example, the estimation that one percent of cancer is due to background ionizing radiation can't be too small by five million times. That wouldn't be very likely.

Clearly, these expressed reservations about the use of quantitative risk assessment reflect the very real weakness in the scientific foundation underlying quantitative risk assessment. This problem should be one of the major priority areas for research related to carcinogen assessment. The regulation of carcinogens is a public health program, and it is difficult to mount a public health program without having some idea of the likely benefits in relation to costs. Hopefully, we will make rapid progress in gaining the knowledge necessary for doing risk assessments with greater confidence.